On the evolution of azole resistance in

Aspergillus fumigatus

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On the evolution of azole resistance in *Aspergillus fumigatus*

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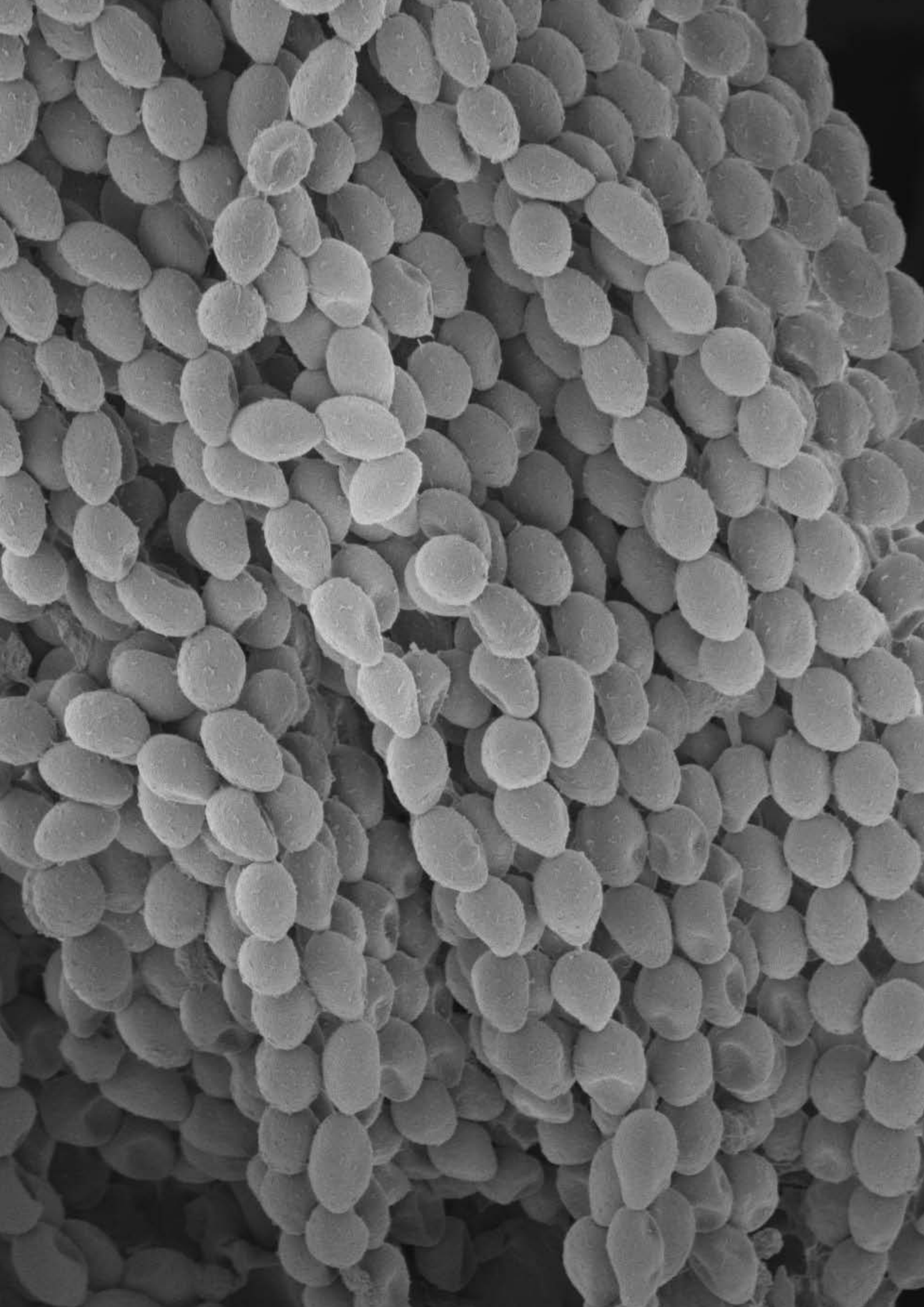

CHAPTER I

General introduction

During the last decade azole resistance has increasingly been reported in *Aspergillus fumigatus,* which is a fungal pathogen involved in the vast majority of invasive aspergillosis infections in humans, and is now a global public health concern (Snelders et al. 2008; Arendrup et al. 2010; Lockhart et al. 2011; Mortensen et al. 2011; Pfaller et al. 2011; Chowdhary et al. 2012; Morio et al. 2012; Gisi 2013; Bignell 2014; Chowdhary et al. 2014; Wiederhold et al. 2015). Antifungal azoles, especially triazoles, are the drugs of choice for medical treatment. However, this treatment is hampered by the emergence of multi-azole resistant *A. fumigatus* isolates, especially the highly resistant variants TR_{34}/L98H and TR_{46} /Y121F/T289A. Therefore, to control this disease, it is essential to elucidate by what mechanisms resistance emerges, how resistance spreads and how resistant genotypes persist in environments without azoles. These research questions are central to this thesis.

This chapter will discuss the current state of knowledge on the factors that are relevant for the development of azole resistance in *A. fumigatus* and its implications for prevention and patient treatment. These factors include the specific biology of *A. fumigatus* and how it affects the mutation supply, natural selection, the possible environments in which resistance can arise (the co-called development routes), the human lung as a suitable habitat for *A. fumigatus*, and the process of adaptation. Finally, this chapter contains an outline of the rest of the thesis.

Aspergillus fumigatus nomenclature

A. fumigatus is a filamentous saprotrophic fungus, and an important family member of the genus aspergillus (Figure 1). It was originally described as an asexual fungus by Fresenius in 1863 and it was named *Aspergillus fumigatus.* In 2009 sexual reproduction was discovered (O' Gorman et al 2009) and *A. fumigatus* was assigned to the genus *Neosartorya fumigatus* based on the similarity of the sexual fruiting bodies to *Neusartorya* (cleistothecia and ascospores) (Rydholm et al. 2006; Samson et al. 2007; O'Gorman et al. 2009).

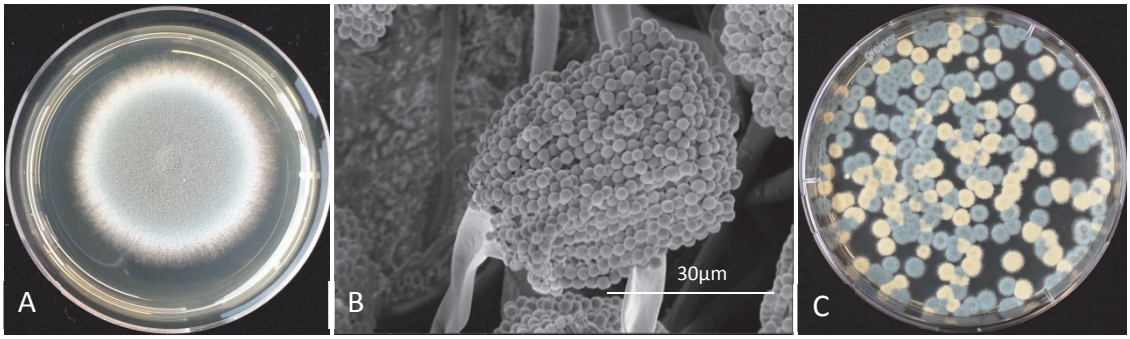

Figure 1. Morphology of *A. fumigatus*

A: Colony of *A. fumigatus* growing on solid medium, B: spore head, C: *A. fumigatus* wild type (Blueish-green) and colour mutant (yellowish) colonies (photos by J Zhang)

General characteristics of growth, life-cycle and genome

A. fumigatus is capable of surviving under a wide range of environmental conditions, such as temperatures of 12-65°C, pH from 3.7 to 7.6 and so on. Surprisingly, the optimal growth temperature of *A. fumigatus* is 37 °C, which is significantly higher than other ascomycetes. Blueish-green conidia (asexual spores) can persist over long periods of time under various (stress) conditions. The hydrophobicity gives spores airborne features that facilitate efficient dispersion – higher than comparable species *A. nidulans and A. niger*. This also makes *A. fumigatus* a challenge to manage during experiments.

Similar to the model species *A. nidulans*, there are various stages in the life cycle of *A. fumigatus* for sexual, asexual and parasexual processes (Figure 2). For *A. nidulans* it has been shown that this life-cycle flexibility can facilitate adaptation and the development of resistance (Schoustra et al 2007). This suggests that specifics on the biology of *A. fumigatus* will have an impact on the development of azole resistance in *A. fumigatus*. For instance, during the large number of mitotic divisions that take place during asexual reproduction, spontaneous mutations have a high likelihood to occur, and might provide the mutational resource for *A. fumigatus* to adapt to azole environment. The process of sexual reproduction shuffles the whole genome (such as recombination as a result of cross overs, gene conversion and so on) and will generate novel genetic variation.

Parasexual processes involve a natural alternation between haploid and diploid states. This may lead to mitotic recombination (crossing-over during mitosis between homologues) and random chromosome loss through repeated non-disjunction can restore the haploid chromosome number. When two different nuclear genotypes occur in a common cytoplasm, recombination by a parasexual process can generate substantial genetic variation and can serve as an alternative for sexual recombination (Pontecorvo et al, 1958).

The full genome of *A. fumigatus* has been sequenced and was first described in 2002 (Denning et al. 2002). Regarding the whole genome, *A. fumigatus* genomes are smaller and substantially less gene rich than genomes of related species *A. oryzae* and *A. flavus* (Gibbons and Rokas 2013). Population genomic analysis of 24 independent sensitive and resistant isolates from the Netherlands and United Kingdom have shown *A. fumigatus* has high genetic diversity based on SNPs screened through whole-genome sequencing (Abdolrasouli et al. 2015).

Ecology of *A. fumigatus*

A. fumigatus is a ubiquitous fungus widely spread in nature, such as in soil, air and especially in decaying plant material, where it plays an important role in carbon and nitrogen recycling. For instance, 100,000 colony forming units per gram can be found in compost (Anastasi et al. 2005; Deacon et al. 2009). In greenhouse and garden soil *A. fumigatus* makes up 35-70 % of the total number of colony forming fungi (Jensen 1931). *A. fumigatus* can also be found indoors, for example in the kitchen, bathroom, basement

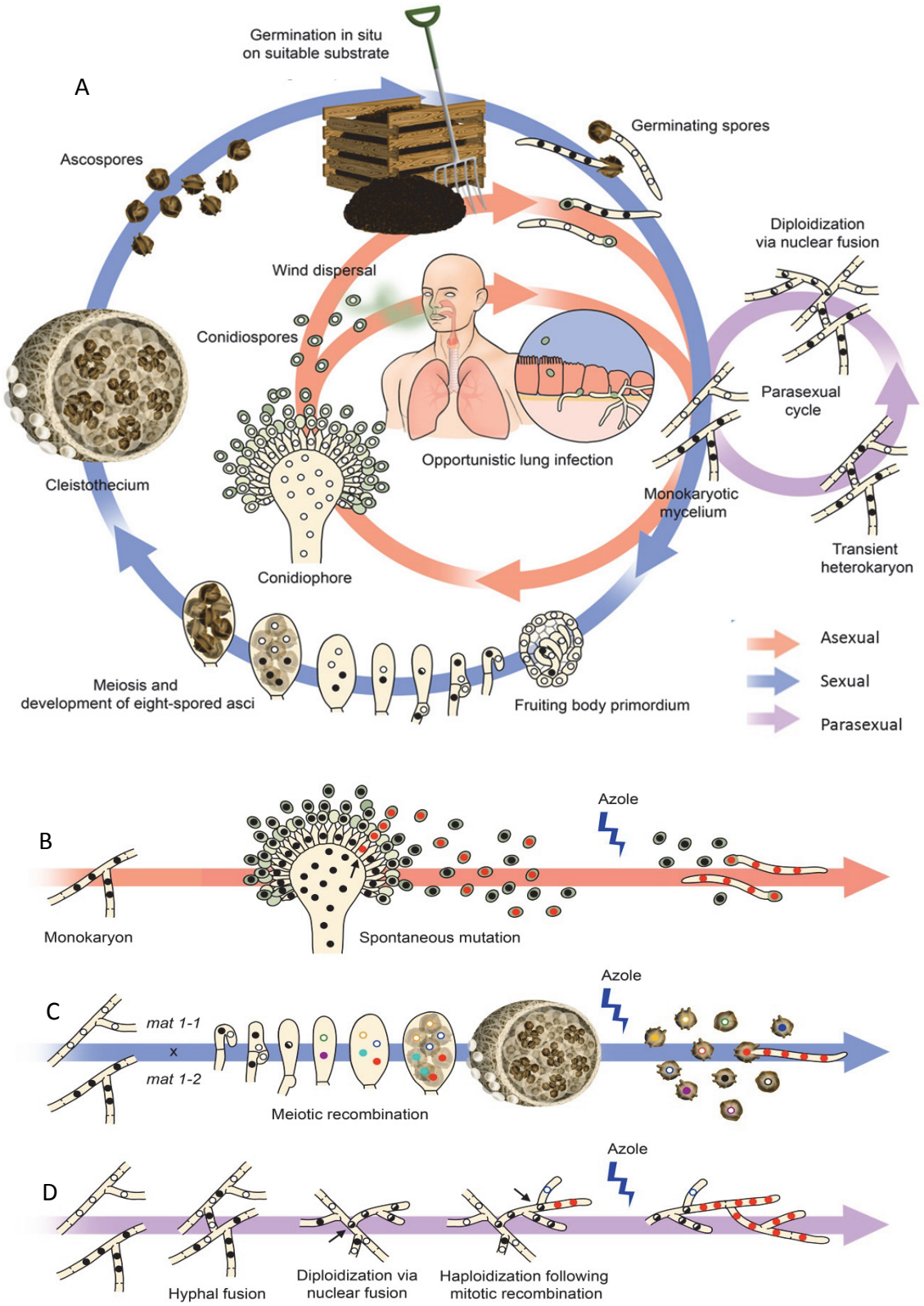

Figure 2. The genotype diversity generated via multiple modes of reproduction in *A. fumigatus*.

A. The life cycles of *A. fumigatus*. B. Formation of conidiospores on aerial conidiophores by repeated mitotic divisions in phiallide cells (~100/conidiophore). Each phiallide divides upto 100 times, therefore each conidiophore contains upto 10^4 spores that are easily dispersed by air (Figure 3). C. Sexual reproduction requires introduction of a male nucleus to a female cell of opposite mating type. Subsequent synchronized divisions of the two nuclei eventually results in

numerous essentially identical meiocytes that undergo meiosis and a post-meiotic mitosis. Each meiocyte thus results in an ascus with eight ascospores. In total a fruiting body (cleistothecium) may contain upto 10^4 asci. D. Mitotic recombination in fungi during somatic growth has been termed parasex. Mycelial growth involves branching and hyphal fusion (anastomosis). Upon hyphal fusion heterokaryotic cells can be formed, if the anastomosing hyphae are of dissimilar genotype. Such heterokaryon may again segregate into homokaryons, but nuclear fusion between different nuclei can also occur. The resulting heterozygous diploid nuclei can segregate into diploid sectors and/or diploid spores. During mitotic division of heterozygous diploid nuclei, mitotic recombination (non-disjunction or crossing over) may occur and result in loss of heterozygosity. Then, upon haploidisation recombinant haploids are formed. Thus the processes of recombination (inter- and intrachromosomal recombination) as observed during the two meiotic divisions also occur during parasex, be it at a lower frequency and less controlled in time in during multiple mitotic divisions. (Drawing by Marc Maas)

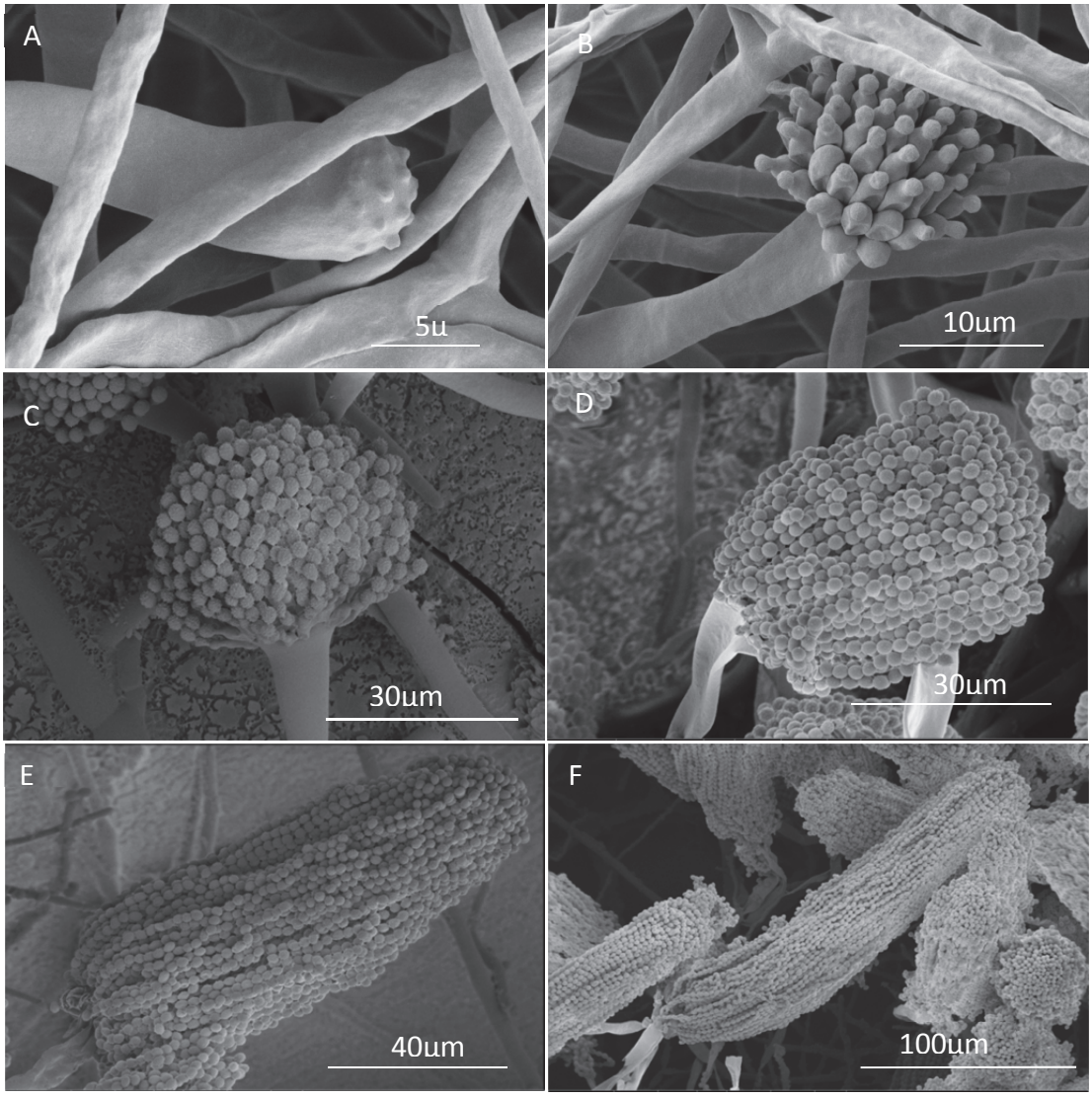

Figure 3. Morphological changes during asexual sporulation in *Aspergillus fumigatus* tracked via scanning electron micrographs

A: Vesicle formation from the tip of the stalk. B: Developed phialides. C: Early stage of conidiophores bearing short-chains of conidia. D: Extended long-chain of conidia. E, F: Mature conidiospores with around 200µm length of chains of conidia. (Photo's J. Zhang)

flower pot soil and so on (Gisi 2013). In air the *A. fumigatus* concentration could easily reach the level of 10-200 conidia per m^3; each day everybody inhales several hundreds of spores.

Diseases caused by *A. fumigatus* in immunosuppressed patients

A. fumigatus is considered an opportunistic pathogen because it only infects immunosuppressed individuals, such as AIDS patients, by inhalation of conidia. Invasive infection in the lung represents a major cause of morbidity and mortality in these individuals (Camps et al. 2012a). Specifically, there is a wide range of diseases related to *A. fumigatus* infection, such as allergic bronchopulmonary aspergillosis (ABPA); invasive aspergillosis (IA); central nervous system invasive aspergillosis (CNS IA); and cavity aspergillus disease (aspergilloma). Different morphs of *A. fumigatus* (spores or hyphae) were detected depending the specific type of disease. In general, *A. fumigatus* can cause disease by growing in the lungs in biofilms/mucus which are places the immune system cannot easily reach (in e.g. cystic fibrosis and COPD - Chronic obstructive lung disease - patients). Upon a total failure of the immune system, it can also spread from the lungs to other parts of the body. In this way, *A. fumigatus* can result in deep-seated infections such as kidney and brain infection, with a patient mortality of 99% (Balloy et al. 2005). In all cases, medical triazoles are the main pharmaceutical treatment tool.

A. fumigatus not only infects immunosuppressed patients, but also domestic animals. It is primarily a respiratory infection, although tissue infected varies among species (https://en.wikivet.net/Aspergillosis).

Several specific features contribute to the pathogenicity of *A. fumigatus.* These include firstly specifics on the host environment (the patient) and secondly specific growth characteristics. Regarding the host environment, several reviews on the pathogenicity of *A. fumigatus* from the perspective of its biology, pathogenesis, molecular biology, and virulence factors suggest *A. fumigatus* requires an immunocompromised host to exhibit pathogenicity (Latgé 2001; Brakhage and Langfelder 2002; Tekaia and Latgé 2005; Hohl and Feldmesser 2007; Dagenais and Keller 2009; Latgé and Steinbach 2009). Only when the immune system of the human host is weak, *A. fumigatus* is exerting as a human pathogen.

Regarding the specific growth characteristics, the following four features are believed to equip *A. fumigatus* for being pathogen (Kwon-Chung and Sugui 2013).

(I) High spore production and effective dispersal of spores by air. After a week of growth, spore production (asexual conidiospores) of an *A. fumigatus* colony could easily reach 10^9. Combined with high hydrophobicity these airborne spores can effectively disperse and could also be transported by swarming soil bacteria or nematodes. In comparison, *A. fumigatus* spores are dispersed much more efficiently than the spores of other fungi.

(II) Physical characteristics that allow conidiospores to reach the distal airways. The small size of conidiospores contributes to respiratory tract diseases. *A. fumigatus*

conidiospores with a size of 2.0-3.5 µm are significantly smaller than those of *A. nidulans* and *A. niger*, can reach the lower airway. Melanin in the conidial wall provides protection from ROS (reactive oxygen species) and lysis in the host cell (Brakhage and Langfelder 2002). Negatively charged sialic acid residues on the conidial surface allows effective binding to the host (Wasylnka et al. 2001).

(III) Survival in a wide range of environmental conditions. A. fumigatus is able to grow in the temperature range of 12-65°C and in acid as well as slightly alkaline environments (3.7-7.6). Sexual ascospores are able to survive a 70°C heat shock for 1 hour. Furthermore, *A. fumigatus* exerts optimal growth and spore production at 37 °C, making the human or other mammalian host a favourable niche.

(IV) Rapid adaptation to changing environments. Plasticity of the *A. fumigatus* genome with rich and diversified gene clusters facilitates *A. fumigatus* to adapt to changing environments. *A. fumigatus* is fully equipped for adjusting gene expression in response to host stress. This allows for adaptation to strong host-defence systems such as the production of potent secondary metabolites (16 metabolites identified) (Frisvad and Samson 1990) efflux pumps, and specific enzymes such as catalases (Burns et al. 2005).

Triazole treatment for *A. fumigatus* infected patients

The ergosterol biosynthesis pathway is the general target for inhibition of fungal growth in the management of *A. fumigatus* infections (Figure 4). There are three types of antifungal agents for treatment of infections: polyenes (amphotercin B), echinocandins (caspofungin) and azoles, of which triazoles are the most widely used in medical treatment, especially itraconazole, posaconazole, and voriconazole (Albarrag et al. 2011). These triazoles inhibit the enzyme sterol 14α-demethylase (Erg11), encoded by the *cyp51* gene, thereby blocking its function of the conversion of lanosterol within the ergosterol biosynthesis pathway, resulting in ergosterol depletion and accumulation of lanosterol which is toxic (Snelders et al. 2012). Itraconazole, clinically licensed in 1997, is commonly used for the treatment of chronic and allergic conditions. Voriconazole, clinically licensed in 2002, is the recommended first choice therapy for invasive aspergillosis and posaconazole, clinically licensed in 2006, is effective in preventing invasive aspergillosis in patients with certain hematologic malignancies (Camps et al. 2012a).

Azole resistance in *A. fumigatus* and resistance mechanisms

A variety of azoles have been used over the years as the main treatment therapy. Over the last decades, azole resistance has increasingly been reported. Notably, if patients are infected with resistant *A. fumigatus*, the mortality associated with aspergillosis is high and the rate of treatment failure is much higher (Howard et al. 2009; van der Linden et al. 2011; van der Linden et al. 2015). The majority of azole resistance is caused by alterations in the target protein sterol 14α-demethylase, encoded by the *cyp51A* gene (Diaz-Guerra et al. 2003; Mellado et al. 2004; Chen et al. 2005; Lockhart et al. 2011).

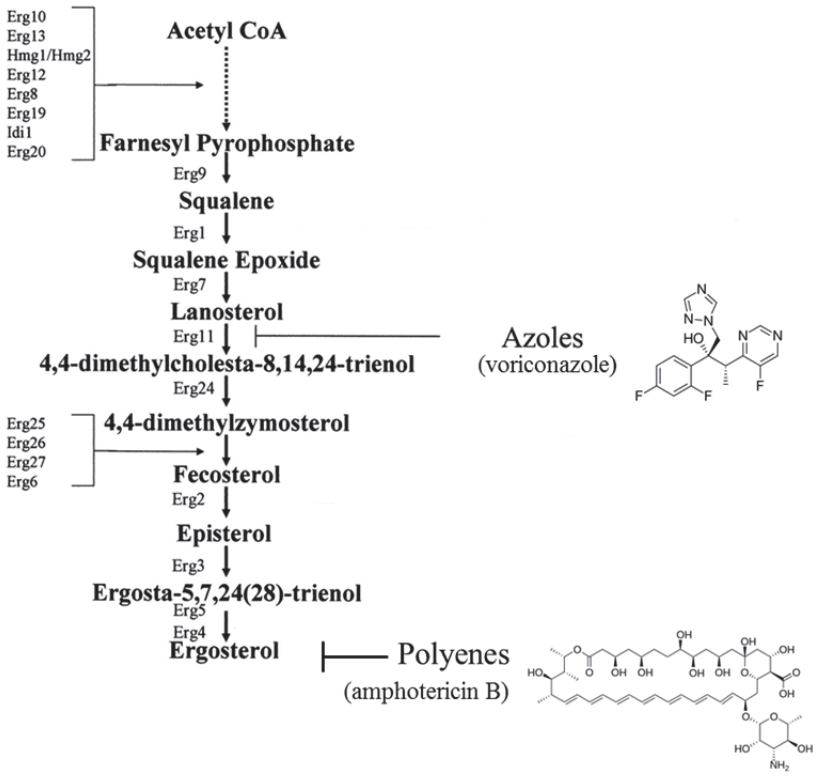

Figure 4. Ergosterol biosynthesis pathway and antifungal targets in *A. fumigatus* (Onyewu et al. 2003).

Point mutations in the coding region of *cyp51A* can lead to structural alterations to the enzyme. Point mutation hotspots in the gene include codons 54 (Diaz-Guerra et al. 2003; Nascimento et al. 2003; Chen et al. 2005) and 220 (da Silva Ferreira et al. 2004; Mellado et al. 2004)(Latgé and Steinbach 2009).

A new resistance mechanism is the combination of a tandem repeat (TR) in the promoter region of *cyp51A* combined with a point mutation in the coding region of the *cyp51A* gene (Figure 5), which was first detected in 1998. The first type was TR_{34}/L98H (the substitution of leucine 98 for histidine, together with the presence of two copies of a 34-bp sequence in tandem in the promoter of the *cyp51A* gene) and was found in the Netherlands in 1998 in the environment and in immunosuppressed patients.

Subsequently, this genotype was found in multiple European Member States, including the United Kingdom, Spain, Belgium, France, Italy, Austria and Denmark, and worldwide (Figure 6) (Snelders et al. 2008; Arendrup et al. 2010; Lockhart et al. 2011; Mortensen et al. 2011; Pfaller et al. 2011; Chowdhary et al. 2012; Morio et al. 2012; Rath et al. 2012; Seyedmousavi et al. 2013; Chowdhary et al. 2014; Wiederhold et al. 2015). TR_{34}/L98H is highly resistant to itraconazole (minimal inhibitory concentration 100% (MIC) > 16), but not to voriconazole and posaconazole. In 2006 another resistant type carrying a 53 bp tandem repeat (TR_{53}) was isolated from a patient with chronic granulomatous disease. This isolate was resistant to itraconazole as well as voriconazole, and showed

reduced susceptibility to posaconazole. Fortunately, for mutants carrying TR_{53}, long term of posaconazole treatment has been shown to still be effective (Hodiamont et al. 2009; Camps et al. 2012c).

The next wide-spread mutant type that appeared was TR_{46}/Y121F/T289A; it first showed up in clinical isolates cultured on 31 December 2009 in Utrecht. From December 2009 to January 2011, 21 TR_{46}/Y121F/T289A *A. fumigatus* isolates were obtained from 15 patients in six different university hospitals in the Netherlands. Soon after, the same mutant type was detected in neighbouring European countries including Belgium (2012), Denmark (2014), France (2015), and outside Europe, for instance in India (2014) (Vermeulen et al. 2012; van der Linden et al. 2013; Astvad et al. 2014; Montesinos et al. 2014; Lavergne et al. 2015; Pelaez et al. 2015; Wiederhold et al. 2015) (Figure 6). In the Netherlands TR_{46}/Y121F/T289A was also found in environmental samples. Although there is variation in the resistance level among these TR_{46}/Y121F/T289A isolates to different triazoles, the strains/genotypes exhibit high resistance to voriconazole, some are highly resistant to itraconazole, and some are highly resistant to posaconazole.

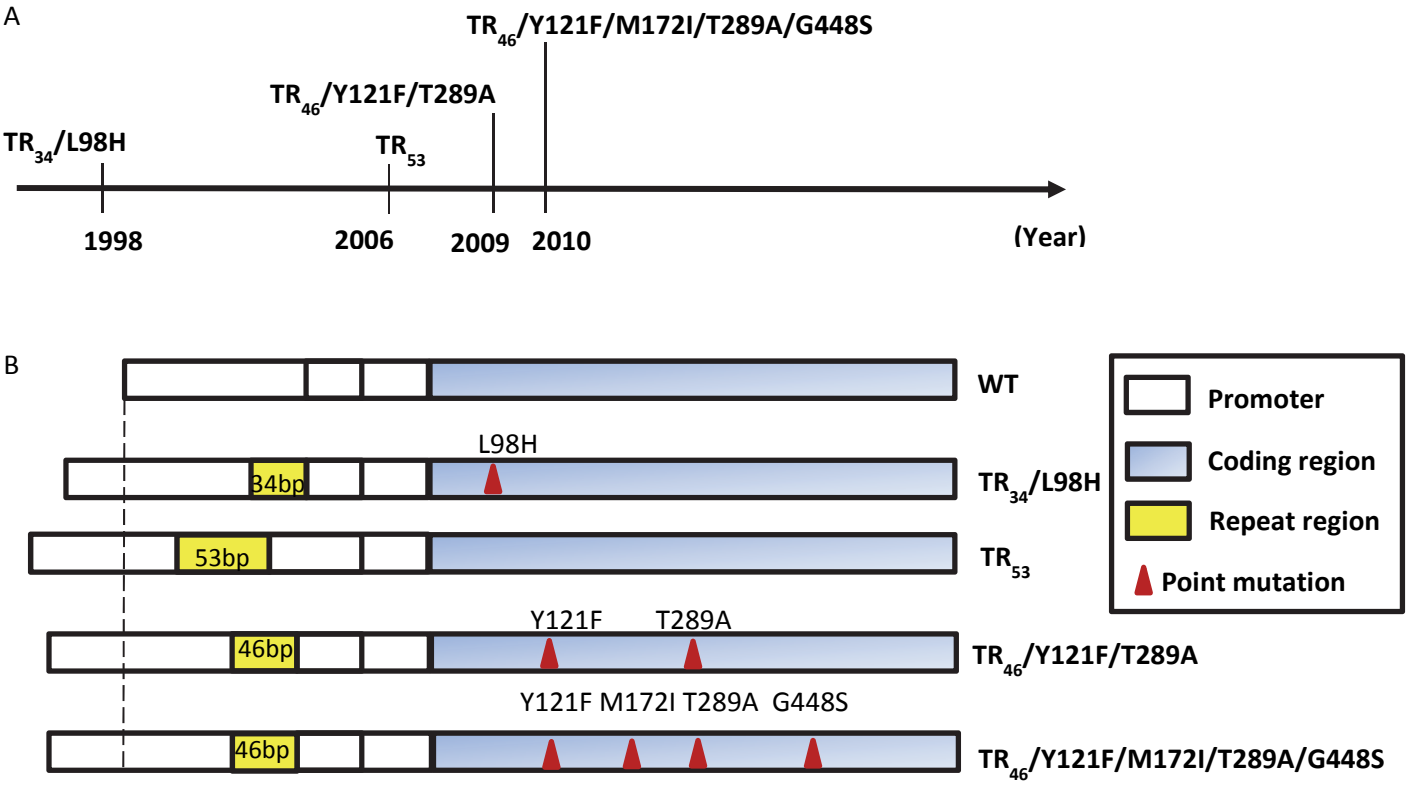

Figure 5. (A) The first year of observation of different azole-resistant mutant types of *A. fumigatus* since 1998 in the Netherlands and (B) illustration of resistant tandem repeat variants on the *cyp51* gene (promoter and coding region)

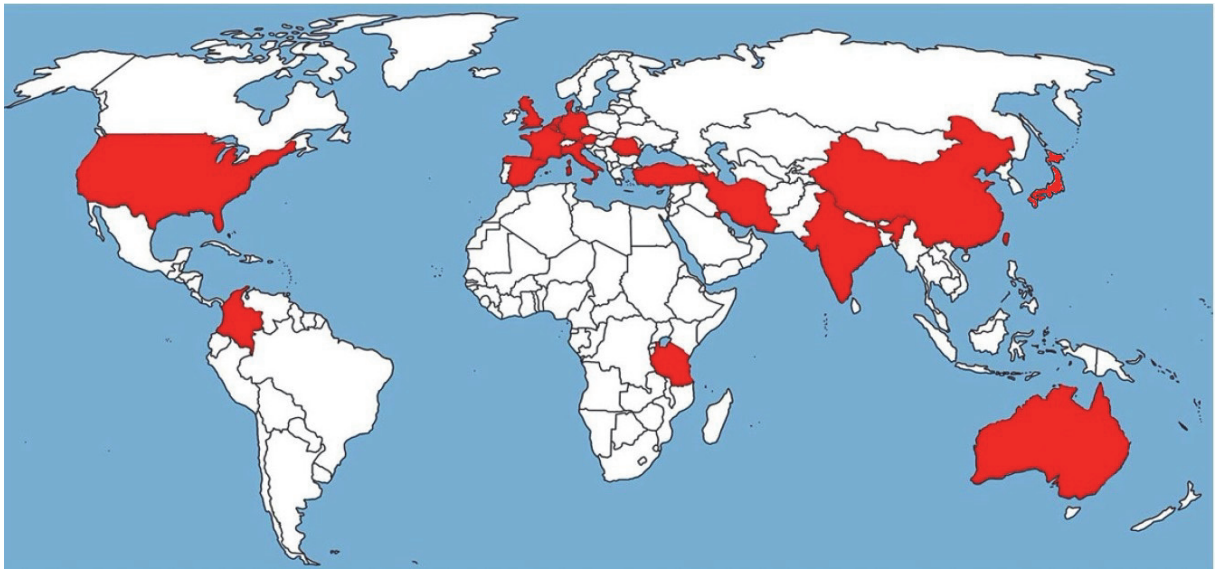

Figure 6. Countries that report the TR_{34}/L98H and TR_{46}/Y121F/T289A resistance mechanism in clinical or environmental *A. fumigatus* isolates in last decade (picture from P Verweij et al., 2015).

Genetic relatedness of TR_{34}/L98H, TR_{46}/Y121F/T289A and the wild-type

Even though TR_{34}/L98H and TR_{46}/Y121F/T289A spread globally, genotyping has revealed high genetic similarity among geographically unrelated strains based on microsatellite locus analysis (Camps et al. 2012b). Especially isolates harboring TR_{46}/Y121F/T289A were found to be genetically highly similar between the Netherlands and India (Chowdhary et al. 2014), while allele-sharing distance matrices of microsatellite genotypes showed that clinical strains carrying either TR_{46}/Y121F/T289A or TR_{34}/L98H were highly genetically different from local wild-type isolates (van der Linden et al. 2013). This information suggests that either the mutations (TR_{34}/L98H or TR_{46}/Y121F/T289A) arose more than once in similar genetic backgrounds, or these mutations arose only once and subsequent variation in microsatellite loci patterns arose by mutation or recombination through sexual reproduction. Either way, this illustrates the need to elucidate what factors are driving resistance development not only for understanding the recent evolutionary history but also for the design of measures for preventing future resistance development.

Resistance development routes

As the resistant isolates are both found in the environment and in patients, it is generally accepted that there are two routes to develop azole resistance in *A. fumigatus*: (1) through selection pressure of azoles from medical drugs in human patients – the clinical route and (2) through the selection pressure from the use of azole fungicides in

agricultural and environment – the environmental route. (Mellado et al. 2007; Howard et al. 2009; Snelders et al. 2009; Arendrup et al. 2010; Mortensen et al. 2010).

(1) Clinical route. Resistance development in the clinic is likely to happen in patients with invasive fungal infection diseases and that are exposed to long-term medical azole therapy. It was found that such therapy rapidly selects for azole resistance (Camps et al. 2012c). Furthermore, it has been argued by Gisi that medical triazoles are 10-100 times more active than most agricultural fungicides for *A. fumigatus* control and potentially exert stronger selection pressure for resistance (Gisi 2013).

*(2) Environmental route***.** Agricultural fungicides are triazole derivatives and abundantly used in our environment for agricultural crop protection (Verweij et al. 2009; Snelders et al. 2012; Stensvold et al. 2012). The volume of azoles and azole-like agricultural fungicides that was used in the Netherlands in 2004 was about 320-times higher than that of azoles used in clinical medicine (about 130 000 kg vs 400 kg) (Verweij et al. 2009). The triazoles are the only class of compounds that are used both in agriculture and in clinical medicine. The volume of triazoles sold has almost doubled between 1995 and 2007 in the European Union.

Support for the idea that clinical resistance can arise in the environment comes from the observation that resistant isolates were found in patients that had never been treated with azoles before diagnosis. In these cases patients likely inhaled spores from (a) resistance isolate(s) from the environment (Verweij et al. 2009). Furthermore, all clinical resistant isolates (TR_{34}/L98H) from the patients were resistant to five agricultural fungicides which strongly supports the environmental route (Snelders et al. 2012). Furthermore, when exposing *A. fumigatus* to the agricultural azole fungicide prochloraz, *A. fumigatus* could develop cross resistance to clinical azoles voriconazole, posaconazole and itraconazole, to tolerance levels that were 256 times higher(Faria-Ramos et al. 2014). Snelders discussed that these agricultural azoles share the same core structure with medical azoles. Due to this similarity they adopt much the same stereometric poses in the active site of *A. fumigatus* as the medical azoles, based on the docking information of these agricultural azoles and 3D-CYP51 of *A. fumigatus* (Snelders et al 2012).

Although there have been no reported cases of an “escape” of a resistant strain from a patient to the environment, both routes are hampering treatment in patients. Given the potentially different selection pressures in both environments, research is needed to identify the driving factors selecting for resistance. Interestingly the widely spread resistant isolates TR_{46}/Y121F/T289A and TR_{34}/L98H have never be generated under laboratory conditions. Therefore, it currently remains unclear what shapes the actual resistance-development routes of these two commonly observed resistance type.

Evolutionary perspective on azole resistance

In essence, the development of resistance to fungicides is an evolutionary process. Fungicides reduce the growth rate (*fitness*) in susceptible types. Genetic variation

generated by spontaneous mutations and recombination followed by natural selection are the evolutionary process by which resistance develops and spreads in populations. This evolutionary process of adaptation can be studied in the laboratory using microorganisms, such as bacteria and viruses as well as unicellular algae and fungi. This type of study is known as experimental evolution and has developed into a successful field of research (Figure 7). This relies on the fact that the microorganisms used have short generation time, allowing many generations in a short time, large populations in a small space, and the possibility to freeze samples for later comparative analysis of evolved and ancestral types. Furthermore, abundant molecular and genomic data, as well as techniques for their precise genetic analyses and manipulation are available (Elena and Lenski 2003). Studying the drug resistance in *Candida albicans* via experimental evolution provides a good example for how to study the development of drug resistance and dynamics of evolutionary adaptation (Cowen et al. 2000; Huang et al. 2011). This illustrates how experimental evolution can be used as a tool to investigate how *A. fumigatus* develops resistance by propagating strains in the laboratory under the exposure of azoles. In these experiments, it is possible to apply various conditions and starting points to study how biological features such as reproduction modes influence the evolution of azole resistance.

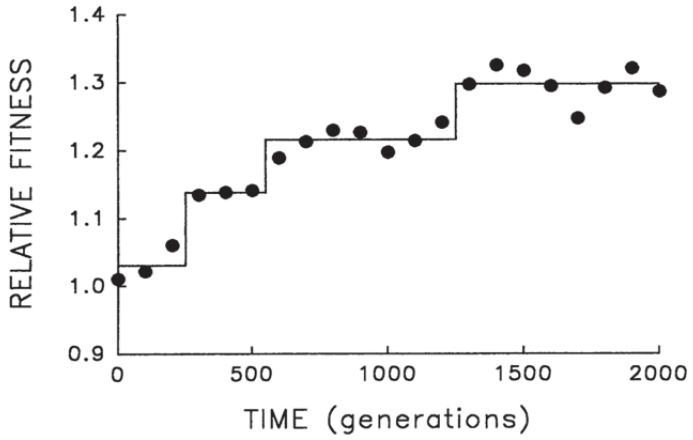

Figure 7. This example of a fitness trajectory shows step-wise increases in fitness, indicating the fixation of beneficial mutations. Over evolutionary time, organisms can increase their fitness by adapting to a certain environment (Lenski and Travisano 1994).

Setup and aim of this thesis

This thesis highlights the relevance of *A. fumigatus* biology and evolution for azole-resistance development and persistence. In this thesis, I firstly exploit the relevance of aspects of the life cycle of *A. fumigatus* for the occurrence of azole resistance in natural

isolates. Next, I develop an experimental-evolution approach to study the long-term dynamics and mechanisms of azole-resistance evolution. In addition, I investigate what condition makes an environment a possible hotspot for the development of resistance. Finally, I link this to the potential conditions under which resistance can emerge and spread in the lungs of humans and how this depends on the specific azole used.

Chapter 2 highlights the current dilemma on the management of patient-acquired resistance in *A. fumigatus*: stopping the use of clinical triazoles or changing to new triazoles? These two scenarios are generally used to control the emergence of azole resistant *A. fumigatus*. On the one hand, after stopping the use of clinical triazoles, resistant *A. fumigatus* may suffer from a cost of resistance due to its adaptation to triazoles making the free-azole environment unfavourable. On the other hand, compensatory mutations may mitigate the initial cost of resistance and aid to retain the resistance phenotype. Therefore, compensatory mutations are required for azole resistance to persist during intermittent use of triazoles in clinics. Similarly, changing to new triazoles will promote the development of additional mutations and could result in pan-resistance to all clinical triazoles. In **Chapter 2**, we provide a patient case with a failure of azole treatment. These cultures exert multiple-phenotypes (with respect to resistance level and morphotypes) over the azole treatment. As the current two treatment solutions, compensatory mutations and additional resistance mutations may arise and contribute to the treatment failure of *A. fumigatus* infection.

Despite the high prevalence of resistant *A. fumigatus* globally, the role of various aspects of the life cycle in the development and persistence of azole resistance has not been studied well. For instance, in clinical practice, in cavitary *Aspergillus* diseases, such as chronic cavitary aspergillosis and aspergilloma, *A. fumigatus* undergoes asexual sporulation, as opposed to invasive aspergillosis, which involves only hyphal growth. This clinical observation also shows that various fungal morphotypes exist within patients. This suggests a link between sporulation and resistance development in patients. In **Chapter 3**, We test the hypothesis that asexual sporulation (one part of asexual life cycle) is required for the spread of azole resistance in *A. fumigatus*, because the asexual sporulation process aids to release the resistant nuclei from multinuclear hypha to single conidiospores, which can regenerate into a fully resistant homokaryotic mycelium. We performed experimental evolution with treatments where the fungus does and does not undergo asexual sporulation. We provide evidence that the cultures that underwent asexual sporulation reached higher resistance levels compared to cultures that did not undergo asexual sporulation. We conclude that asexual sporulation indeed plays an important role in the emergency of azole resistance development. We demonstrate that azole resistance is not fully dominant but partially recessive, which is in line with the hypothesis that asexual sporulation is required to help the resistance nuclei escape from the multicellular hypha. In addition, we put forward an additional hypothesis that asexual sporulation could increase mutation load due to the large number of mitotic division involved in the asexual sporulation process. This has

implications for the increase in resistance over treatment in the cavities of aspergillosis patients, where asexual sporulation takes place.

In **Chapter 4,** we explore the potential of two possible routes of azole-resistance development, through azole exposure in clinics and the environment. While the environmental route has been suggested to be important, there is no direct evidence of the development of clinical resistance via this route. We apply experimental evolution and exposed a sensitive ancestor to five agricultural fungicides that have been used the past 30 years. We examine whether highly resistant isolates or isolates with the commonly found TR_{34}/L98H resistance mechanism emerged and whether these isolates developed cross resistance to medical azoles. The patterns of evolutionary trajectories of evolving strains assayed on medical and agricultural azoles show high similarity. This supports the environmental route: the development of clinical resistance can emerge through the exposure to agricultural azoles. We observe dynamic changes in phenotype and genotype over the nine weeks of the experiment, which shows also within lineages evolved isolates differ in resistance level, growth rate, colony structure and time of sporulation. Even though no TR_{34}/L98H mutants were discovered, we find one mutation in the *cyp*51 gene (G138S) in one out of 36 lineages. Lastly, potential candidate genes that are related to resistance mechanism were investigated after whole-genome sequencing.

Chapter 5 follows up on the observation of different morphotypes occurring within evolving lineages in the evolutionary experiment of **Chapter 3,** as well as in samples obtained from clinical isolates of single patients, which suggests that morphology of isolates may play a role in azole-resistance development. We investigated whether these isolates with different morphotypes are vegetatively compatible and considered the relevance of heterokaryon formation in the development of azole resistance in *A. fumigatus*. Heterokaryon compatibility tests show that all morphotypes isolated from the same patient are vegetatively compatible, while isolates taken from different patients are not. This indicates that different morphotypes from one patient likely originated from one original isolate and that diversification takes places within the patient in response to selection. Furthermore, we show how the flexibility of the heterokaryon aids adaptation to changing azole environments, which promotes the persistence of azole resistance in *A. fumigatus*.

A. fumigatus is a ubiquitous fungus. However, the hotspot for resistance development in the environment is unknown. Further, it has been suggested that sexual reproduction can aid resistance development; evidence for this notion is also still lacking. In **Chapter 6,** we investigate two compost–heaps, one containing and one not containing azoles. We find a high incidence of *A. fumigatus* isolates in both, but a larger genetic diversity of resistant *A. fumigatus* within azole-containing compost than within azole-free compost. This suggests that azole-containing compost is a hotspot for the development and maintenance of azole resistance in *A. fumigatus*. Importantly, we find a novel resistance mechanism with $TR_{46}{}^{3}$/ Y121F/ M172I/T289A/G448S, a type that is resistant to all

available medical triazoles. Upon investigation, this novel mutation is also present in several patients from different hospitals in the Netherlands. The existence of two mating types among the isolates containing the TR_{46}^{3}/ Y121F/ M172I/T289A/G448S mutations and the possibility to generate TR_{46}^{3}/ Y121F/ M172I/T289A/G448S during a sexual cross with two strains containing TR_{46} suggest that the occurrence of sex in compost is highly likely. However, more evidence is needed. This finding illustrates how rapid development of resistance in *A. fumigatus* could arise and further supports the presumed possible role of sexual reproduction in the development of azole resistance.

In **Chapter 7**, I put the results attained through the whole thesis in a broader context and I highlight the relevance of these findings to *A. fumigatus* infections from both the clinical perspective as well as the perspective of fungicide management in agriculture.

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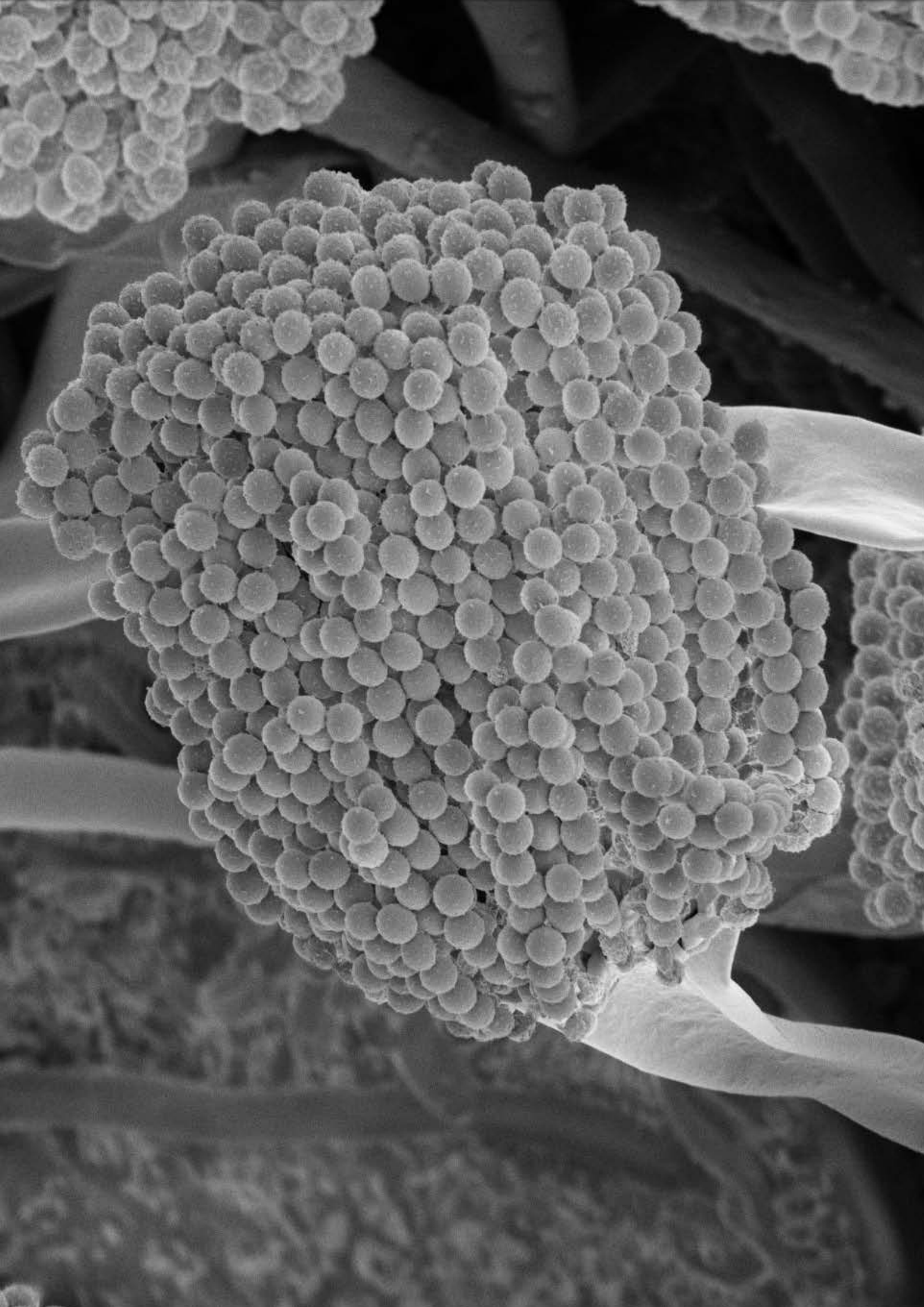

CHAPTER II

In-host adaptation and acquired resistance in *Aspergillus fumigatus*: on the horns of a dilemma

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Abstract

Aspergillus fumigatus causes a range of diseases in humans, some of which are characterized by fungal persistence. *A. fumigatus* may persist by adapting to the human lung environment through physiological and genomic changes. The former is based on the large biochemical versatility of the fungus, the latter on its capacity to generate genetic diversity by spontaneous mutations and recombination, and subsequent selection of genotypes most adapted to the new environment. Stress factors such as triazole exposure causes mutations to emerge that render resistance. The mode of reproduction, i.e. sexual, parasexual, or asexual, is probably crucial for the adaptive potential of *Aspergillus*. As any change in the environment may provoke adaptation, switching between triazoles in patients with chronic pulmonary aspergillosis (CPA) may result in a high-level pan-triazole-resistant phenotype through the accumulation of resistance mutations. Alternatively, when triazole therapy is stopped, an azole-free environment is created that may prompt selection for compensatory mutations that overcome any fitness costs that are expected to accompany resistance development. As a consequence, starting, switching, and stopping azole therapy has the risk of selecting for highly resistant strains with wild-type fitness. As similar adaptation is expected to occur in response to other stress factors such as endogenous antimicrobial peptides, over time the fungus will become increasingly adapted to the lung environment thereby limiting the probability of eradication. Our hypothesis challenges current management strategies and future research should investigate the genomic dynamics during infection in order to understand the key factors facilitating adaptation of *Aspergillus*.

Introduction

Aspergillus fumigatus is a ubiquitous fungus that plays an important role in carbon and nitrogen recycling in nature by degradation of organic biomass.[1,2] The most well-known habitat is the compost pile, which is considered a harsh and competitive environment.[3-5] Various biological characteristics allow *A. fumigatus* to thrive in this environment, including rapid and efficient germination, growth at elevated temperatures, and a metabolism that is responsive to variation in nutrient sources.[6] Furthermore, *A. fumigatus* propagates via asexual spores that can be dispersed over large geographic distances by air currents and that can germinate to grow under a broad range of environmental conditions.[7-9] This remarkable biochemical versatility of *A. fumigatus* contributes to its role as the predominant fungal pathogen of immunocompromised patients.[10] Several diseases may be caused by *A. fumigatus* ranging from allergic conditions to acute invasive aspergillosis (IA).[11] Furthermore, in order to survive and thrive *Aspergillus* needs to rapidly adapt in these environments that often entail various challenges.

Genetic adaptation can be defined as the acquisition of heritable modifications in an organism through natural selection that enable it to survive and reproduce in the prevailing or new environment.[12-15] One example of this process of adaptation is the development of azole resistance. Triazoles have become the cornerstone of prevention and treatment of *Aspergillus*-related diseases.[16] Although triazole resistance may develop during patient therapy,[17,18] the main route of resistance selection in *A. fumigatus* is believed to be through exposure to azole fungicides in the environment.[19] Azole compounds are widely used for food production, plant protection, and material preservation.[20,21] Patient-acquired resistance and environmental resistance both require an active reproducing fungus and exposure to azole compounds. We hypothesize that *A. fumigatus* is capable of adapting to the human host during infection, and that adaptation is an important fungal property that contributes to treatment failure and persistence of the fungus. Here, we review adaptation strategies of *A. fumigatus* in relation to azole-resistance-selection and the clinical implications thereof for management of *Aspergillus* diseases.

Adaptation strategies in *A. fumigatus*

Three modes of reproduction are recognized in *A. fumigatus*, namely asexual, sexual, and parasexual reproduction (Figure 1A). In contrast to sexual and asexual reproduction that are known to widely occur in many microorganisms, parasex is more a fungal strategy to generate genetic diversity than a reproduction mode. The consequence of these aspects of the lifecycle is an increased genetic diversity, through spontaneous mutations and reshuffling of genotypes, thereby increasing the likelihood that the offspring includes types that are better adapted to a new environment. Azoles are not known to be mutagenic or recombinogenic *per se*, but exposure of *A. fumigatus* will provoke a strong selection pressure for azole resistance.

When conditions are right, a fungal colony is initiated from the germination of a single spore that will give rise to a network of hyphae. After a few days, when vegetative hyphae are exposed to air, aerial hyphae form asexual reproductive structures, conidiophores, that each produce up to 10^4 asexual spores.[22] Consequently, after one week a colony may have produced up to 10^9 spores that easily become air-borne. During each mitosis there is a very small probability of a mutation arising. However, given the large number of mitotic divisions that take place during asexual reproduction, asexual spores provide a large supply of spontaneous mutations e.g. for new azole resistance mutations to be selected in the azole environment (Figure 1B).[23]

Sexual reproduction requires two parental strains of opposite mating type (MAT1-1 and MAT 1-2).[24] After fertilization, a fruiting body (cleistothecium) is formed that may contain 10^4 sexual spores that originated from the same zygote and that are genetically different due to recombination during meiosis. Genetic variation that is present in the parental strains is thus enhanced through the reshuffling of these genotypes. Genes and alleles associated with low-level resistance might thus evolve to a high(er) resistant phenotype when placed in a different genetic background (Figure 1C). Persistent and/or increased azole pressure will facilitate the emergence of such high-level resistant phenotypes through natural selection. Sexual reproduction, which has thus far been demonstrated only under specific laboratory conditions, can take several months to complete.[17,24-27] It is believed that the sexual stage is the main source of increased variation in fungi, but in *A. fumigatus* it has not yet been demonstrated in nature (Table 1).[28]

Parasexual recombination also contributes to increased genetic variation through reshuffling of genes, which may occur in genetically different, but compatible hyphae. Fusion of hyphae allows the nuclei to fuse as well, resulting in a temporally diploid phase where mitotic recombination may occur, before reverting to the normal haploid stage (Figure 1D).[29,30] Again, existing genetic variation is thus increased enabling the production of genotypes that are better adapted to a new environment.

Patient-acquired resistance to azoles

In addition to the above-mentioned fungal characteristics, properties related to the drug are important with respect to resistance development. Rapid fungicidal drug activity might reduce the ability of fungi to adapt. *In vitro* experiments indicate that the polyene amphotericin B is fungicidal against most fungi, including *A. fumigatus*.[31,32] Acquired resistance against polyenes is extremely rare, despite the use of this class in clinical medicine for over 50 years.[31] Triazoles show differential fungicidal activity against *Aspergillus* species, and are commonly fungistatic.[32] In one study voriconazole exhibited fungistatic activity against 15 of 16 (94%) clinical isolates.[32] *A. fumigatus* may thus survive in the azole-environment, thereby enabling adaptation to occur. However, *in vitro* conditions do not simulate well the *in vivo* microenvironment, with fluctuating drug concentrations and the presence of effectors of the innate immunity and serum

Reproduction mode	Fungus characteristics	Environmental conditions	Presumed Benefit	Likelihood in human host
Asexual	• Mycelium + asexual spores	• Abundantly present in nature • Presence of air/oxygen • Presence of light	• Ability to produce prolific numbers of asexual spores for dispersal and increased mutation supply on a wide range of substrates under a broad set of environmental conditions	• May occur in a lung cavity; chronic cavitating pulmonary aspergillosis, aspergilloma, or sinus disease
Sexual	• Requires opposite mating types (MAT1-1 and MAT1-2)	• Only reported under laboratory conditions • Oat meal agar • 30°C • Darkness • Occurs after 4 weeks or longer	• Long term survival strategy • Heat resistance • Generation of genetic variation for adaptation	• Unlikely due to required conditions
Parasexual (heterokaryon)	• Mycelium containing different compatible genotypes	• Unknown • Expected to occur in long - lasting mycelium	• Genetic recombination and complementation	• May occur in conditions with long-lasting biofilms harboring various *A. fumigatus* genotypes: aspergilloma, sinusitis and chronic colonization (CF)

Table 1. Modes of reproduction in *A. fumigatus*, their characteristics and probability of occurring in the human host

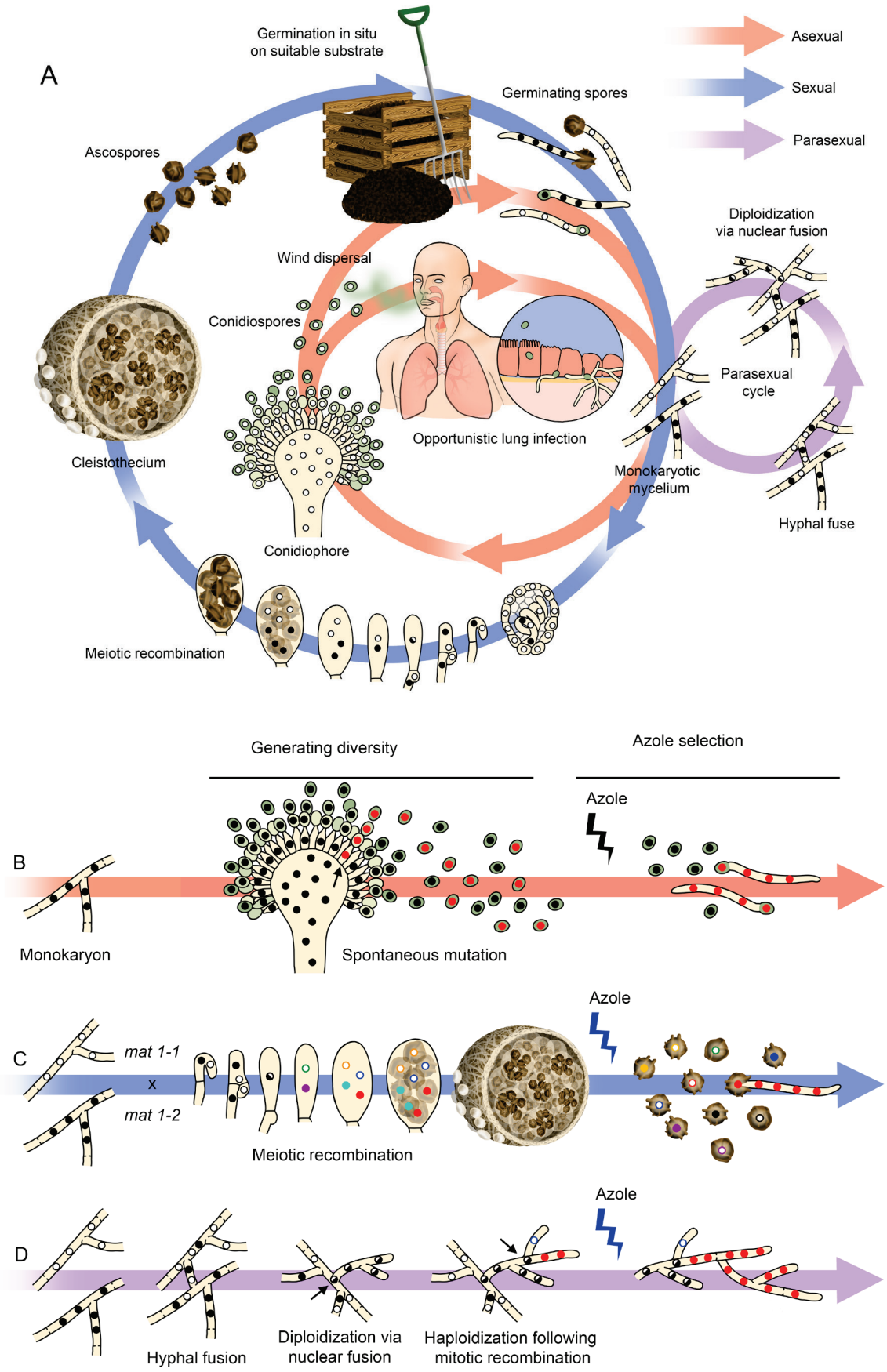

Figure 1. Reproduction modes in *A. fumigatus* and adaptation strategies in response to azole exposure.

Panel A, Sexual reproduction (blue line); asexual reproduction (orange line), and parasexual recombination (purple line). Panel B: Asexual reproduction increases spontaneous mutation supply

from mitotic divisions during the production of numerous conidiospores that are produced. Rare resistance mutations may be selected for when exposed to azoles. Panel C: sexual reproduction as an important strategy to enhance genetic diversity in fungi through meiotic recombination. Panel D: parasexual cycle, which relies on heterokaryons that may arise by mutation or fusion of genetically different but compatible hyphae and subsequent nuclear fusion. This allows (mitotic) recombination to occur in the transient diploid stage, which results in increased genetic variation. Whether the presence of azoles in the environment influences these life stages by for instance increasing the mutation frequency or the frequency of any of the reproduction modes (early in the process) is unclear, but it will provide strong selection pressure after resistant phenotypes have emerged. Black indicates wild-type, and red azole-resistant nuclei. Other colors represent genetic variability of the nuclei.

proteins.[33] The fungicidal activity of voriconazole increased when tested in the presence of serum, [33] underscoring the more complex dynamics during infection in the host. Azole-resistance selection has been observed in patients during azole therapy. In 154 patients, with mainly chronic pulmonary aspergillosis (CPA), a relationship was found between triazole-treatment history and *A. fumigatus* triazole MIC.[34] The itraconazole-dosage duration positively correlated with the itraconazole MIC ($r=0.5700$, $P<0.0001$), and all itraconazole-resistant isolates (MIC > 2 mg/l) were cultured from patients that had been exposed to itraconazole for more than 115 days.[34] However, recovery of azole-resistant *A. fumigatus* during azole therapy could originate through two routes. The patients could inhale azole-resistant airborne spores that are present in the environment, or azole-susceptible *A. fumigatus* might develop azole-resistance mutations during infection in the host.[35,36] Azole-resistance mutations that are associated with the environment commonly consist of alterations in the *cyp51A* target gene combined with transcriptional enhancers in the promoter region.[20,36] Patient-acquired resistance is characterized by point mutations in the *Cyp51A*-gene or unknown resistance mechanisms.[18] However, both environmental and patient-acquired resistance mutations can be found in azole-treated patients.[18,37] Patient-acquired resistance can be proven when a switch in phenotype is observed via the culturing of isogenic isolates from consecutive respiratory samples.[17,38-41] In a review of eight patients with documented conversion of wild-type to triazole-resistant *A. fumigatus*, the time between the last culture of an isolate with an azole-susceptible phenotype and the first isolate with an azole-resistant phenotype varied between three weeks to 23 months (median, four months).[17] Furthermore, in the majority of patients with patient-acquired resistance, multiple azole-resistance mechanisms emerged during therapy.[17,18,34] As many patients with CPA remain culture negative, molecular detection of Aspergillus and resistance mutations directly in respiratory secretions provides further insight in resistance development. For instance, in one study *Aspergillus* DNA was detected in 15 of 19 patients (78.9%) with allergic bronchopulmonary aspergillosis (ABPA), while all patients were culture negative.[37] In CPA patients *Aspergillus* PCR was positive in 71.4% of patients, compared to 16.9% who were *Aspergillus* culture-positive.[37] In six of eight (75%) ABPA patients and 12 of 24 (50%) with CPA resistance markers were detected by PCR. In four patients cyp51 point mutations were found compared to 16 patients who harbored the combined TR_{34}/L98H mutation, indicating patient- and environment-acquired resistance, respectively.[37]

The risk for patient-acquired resistance appears to be associated with a lung cavity, i.e. chronic cavitary pulmonary aspergillosis or an aspergilloma.[17,18,38-40] Histologically, aspergillomas show layers of hyphal growth with a variably dense-layered appearance consistent with extracellular matrix and extracellular DNA, representing an extreme form of biofilm.[42] Sometimes conidiophores are present, which might be a key factor for the development of resistance mutations. Indeed, *in vitro* studies showed that the azole-resistance level in *A. fumigatus* gradually increased when the fungus was exposed to azole fungicides and underwent asexual sporulation.[23] On average, resistance levels were twice as high compared to the same isolates exposed under non-sporulating conditions.[23] This is explained by the fact that asexual sporulation provides the opportunity for mutations to be released from the non-resistant mycelium and to fully express their phenotype into a newly formed mycelium founded by this resistant single uninucleate spore (Figure 1B).[23] Moreover, asexual sporulation greatly increases the population size allowing for many more possible mutants to be exposed to (azole) selection pressure by increasing the mutation supply through the numerous additional mitoses required to generate all the spores.[23] *A. fumigatus* isolates recovered from sputum of patients with aspergillomas often exhibit a highly atypical macroscopic morphology in culture, with slow growth and poor sporulation.[43,44] Microsatellite genotyping of multiple triazole-susceptible and triazole-resistant *A. fumigatus* colonies derived from dissected aspergillomas indicated extensive genetic changes.[42] Both phenotypic and genotypic observations suggest a remarkable ability of *A. fumigatus* to change within the lung environment.

Unlike in cavitary *Aspergillus* disease, the histology of invasive aspergillosis (IA) shows *A. fumigatus* growing in tissue exclusively through hyphal elongation. As the fungus cannot benefit from one of the above-mentioned reproduction modes, the ability to increase genetic variation is restricted and consequently the risk of azole-resistance development is significantly reduced. Consistent with this notion patient-acquired resistance in IA has not been reported, even in triazole-exposed patients. Long-term prophylactic exposure to posaconazole in 22 lung-transplant recipients colonized with *A. fumigatus* or with IA showed no evidence for the emergence of resistance.[45] The cases of triazole resistance in this patient group are all associated with environmental resistance selection, where *Aspergillus* spores already resistant to azoles are inhaled, rather than resistance selection during therapy.[46,47]

In cystic fibrosis (CF), patients may be chronically colonized with *A. fumigatus*. Genotyping indicates that both identical and variable genotypes may be found in sputum cultured over multiple years.[48] Triazole resistance in CF has been investigated in several studies. An azole-resistance frequency of 3.4% (4 of 119 colonized CF patients) was reported in Germany,[49] 4.6% (6 of 131 patients) and 8% (4 of 50 patients) in France,[50,51] and 4.5% (6 of 133 patients) in Denmark.[48] Several studies indicated that resistance or increased resistance frequency was associated with itraconazole therapy.[48,50] The resistance mutations that are found in CF suggest that both environmental- and patient-acquired resistance plays a role.[48-51] However, it remains unclear how patient-acquired

resistance develops in CF, i.e. which reproductive mode is involved as pulmonary cavities are rarely present. In CF it is thought that *A. fumigatus* forms hyphal networks that resemble biofilm structures,[52] where the fungus may be captured in a hyphal state. As the lungs typically harbor genetically diverse *Aspergillus* spores,[53] this may be a condition where parasex can take place, although this has not been investigated. In IA genetically diverse aspergilli may also be present in the lung,[54] but the spores evolve into individual nodular lesions, thus precluding interaction between genetically different hyphae.

Adaptation to other environmental factors

Recent studies indicate that fungi including *A. fumigatus* sense the host's immune environment.[55,56] Interleukin-17A is the characteristic cytokine of the T-helper 17 (Th17) response that is important for anti-*Aspergillus* host defense.[57,58] In addition to its role of recruiting neutrophils to the site of infection and the induction of antimicrobial peptides, IL-17A can also augment adhesion and filamentous growth of *A. fumigatus* itself, resulting in enhanced biofilm formation and resistance to local antifungal defenses.[55] Furthermore, *in vitro* models indicate that *A. fumigatus* becomes increasingly resistant to polyene, azole, and echinocandin antifungal agents throughout morphological differentiation, with the lowest effects against *A. fumigatus* that had formed an extracellular matrix.[59] In addition, effectors of the innate immunity such as antimicrobial peptides, which are induced in bronchial epithelial cells in response to *A. fumigatus*, exhibit antifungal activity.[60] Adaptation through biofilm formation or possibly antimicrobial-peptide-resistance mutations will further enable the fungus to persist in the host.

Fitness cost and compensatory mutations

It is generally expected that resistance mutations come with a cost: in the absence of the antifungal, the resistant genotype has a lower fitness than wild-type isolates.[61,62] Due to this fitness cost, the resistant type may disappear when in competition with wild-type isolates in antifungal-free environments. However, *A. fumigatus* triazole-resistance mechanisms that are associated with the environment, i.e. TR_{34}/L98H and TR_{46}/Y121F/T289A, have no apparent fitness cost and have been successful in survival and migration in the environment.[63,64] There are several hypotheses that might explain the lack of a fitness cost: (i) the resistant strains may show this cost in specific environments alone, and so far strains have only been tested under optimal laboratory conditions, (ii) TR_{34}/L98H and TR_{46}/Y121F/T289A may have already developed mutations that compensate for any fitness cost by exposure to an azole-free environment (compensatory evolution; see below), and (iii) the tandem repeat (TR) in the promoter region might be the compensatory mutation for the point mutations present in the *Cyp51A*-gene.

When wild-type *A. fumigatus* is cultured in the presence of an azole, the colony will grow at a much slower rate than in the azole-free environment (Figure 2). Following prolonged azole exposure a sector may appear due to the outgrowth of *A. fumigatus* that

has acquired azole resistance and thus has an increased growth rate in the presence of the azole. However, when this azole-resistant colony is transferred to an azole-free environment, the growth rate is lower compared to the original wild-type isolate. The reduced growth rate is considered the fitness cost associated with the acquisition of azole resistance. Continued incubation in the azole-free environment shows the emergence of one or multiple sectors with an increased growth rate (Figure 2). These sectors contain *A. fumigatus* that has (partly) overcome the fitness cost due to compensatory mutation(s). The isolate remains azole-resistant, but now has high fitness both in azole-containing and azole-free environments. Similar observations have been reported in *A. nidulans* when exposed to the fungicide fludioxonil.[62] Thus, alternating between growth conditions with and without the fungicide resulted in a growth rate that was actually greater than that of the original isolate.[62]

Comparably, triazole-resistant *A. fumigatus* cultured from triazole-treated CPA-patients may show an abnormal growth rate or sporulation when brought from an azole-exposed lung tissue to an azole-free agar plate; starkly different conditions that are likely to reveal any resistance-associated fitness cost.[43] However, in such azole-free environments, the resistant genotype may regain fitness by accumulating mutations that decrease the fitness disadvantage while resistance remains unaffected.

Importantly, when the concept of compensatory evolution is extrapolated to *Aspergillus* infections, stopping triazole therapy would be an incentive for resistant *A. fumigatus* to accumulate compensatory mutations that overcome any potential fitness costs. As *A. fumigatus* is able to adapt to an azole-free environment under laboratory conditions, it seems likely that this might also occur in the human lung during infection or colonization. This hypothesis is currently unexplored in *Aspergillus* diseases, but its potentially significant implications for patient disease management warrants research into these aspects of adaptation.

A clinical example

A 36-year-old male diagnosed with X-linked chronic granulomatous disease was admitted to the hospital due to swelling and pain of the right clavicula without previous trauma. The patient had a long history of *A. fumigatus* diseases including aspergilloma and three episodes of IA. He had developed severe chronic obstructive pulmonary disease (Gold IV) and allergic bronchopulmonary aspergillosis. He later developed a second aspergilloma, which could not be surgically removed due to his poor respiratory condition. The patient received prophylaxis with interferon-gamma, trimethoprim-sulphamethoxazole, and itraconazole. CT-scan showed a subcutaneous abscess with involvement of the sterno-clavicular joint. Tissue biopsies confirmed the presence of *A. fumigatus*. Between 2011 and 2013 the patient was treated with various antifungal regimens including switching between various azole drugs, combination therapy or polyene-based therapy (Figure 3). Despite these efforts, eradication of the fungus was not achieved and the patient died. Between 2011 and 2013 thirteen *A. fumigatus* isolates were cultured with various, often atypical, macroscopic phenotypes (Figure 3), while

genotyping using microsatellite markers showed the same genotype in all 13 isolates.[54] Analysis of the azole-resistance phenotype and genotype, showed increasing resistance to the medical triazoles over time, ultimately resulting in a high-level pan-triazole-resistant phenotype (Figure 3). The variation of the colony morphology suggests that azole resistance was accompanied with a fitness cost, i.e. the fungus had adapted to growth in the azole-exposed lung environment, and showed difficulty in growing in the absence of azoles. However, the phenotypes in terms of growth rate and sporulation were highly diverse, suggesting that some strains were better adapted to growth in the absence of azoles than others.

On the horns of a dilemma

We hypothesize that *A. fumigatus* is capable of efficiently adapting to the lung environment through the successive acquisition and selection of mutations that increases its fitness in the new environment. As the fungus is exposed to many stress factors, including antifungal azoles and effectors of the innate immunity, the generation of new mutations and subsequent natural selection will facilitate that it can overcome these factors. The mode of reproduction is probably important, with clinical and experimental data supporting a pivotal role for asexual reproduction. Switching between triazole compounds is recommended in the management of CPA in patients who are intolerant or fail to other azole compounds.[11,65] However, this approach might lead to accumulation of new resistance mutations, ultimately resulting in a high-level pan-triazole-resistant phenotype. Patients with high-level pan-triazole-resistant *A. fumigatus* have been reported, some with a history of multiple-triazole-treatment regimens.[66] Switching between triazoles will ultimately be ineffective if eradication of *Aspergillus* is not achieved (Figure 4).

Moreover, experimental evolution indicates that when *A. fumigatus* is brought into an azole-free environment, compensatory mutations might occur that overcome any fitness costs associated with resistance development (Figure 2). Triazole therapy is commonly stopped in patients when azole resistance is documented and clinical experts recently recommended moving away from triazole monotherapy in azole-resistant *Aspergillus* disease.[67] In patients with CPA this approach may result in gain of fitness of the fungus, especially in case repeated courses of triazoles are given and again stopped (Figure 4). The presence and clinical implications of compensatory evolution in *A. fumigatus* is largely unknown. Mutations associated with the patient route of resistance development, M220 and G54, have recently been recovered from the environment.[68,69] Although it is unclear whether these mutations arose through hospital azole use, any fitness cost could have been overcome by compensatory evolution thus allowing survival in their natural environments.

The two above-mentioned scenario's lead to outcomes neither of which are desirable for

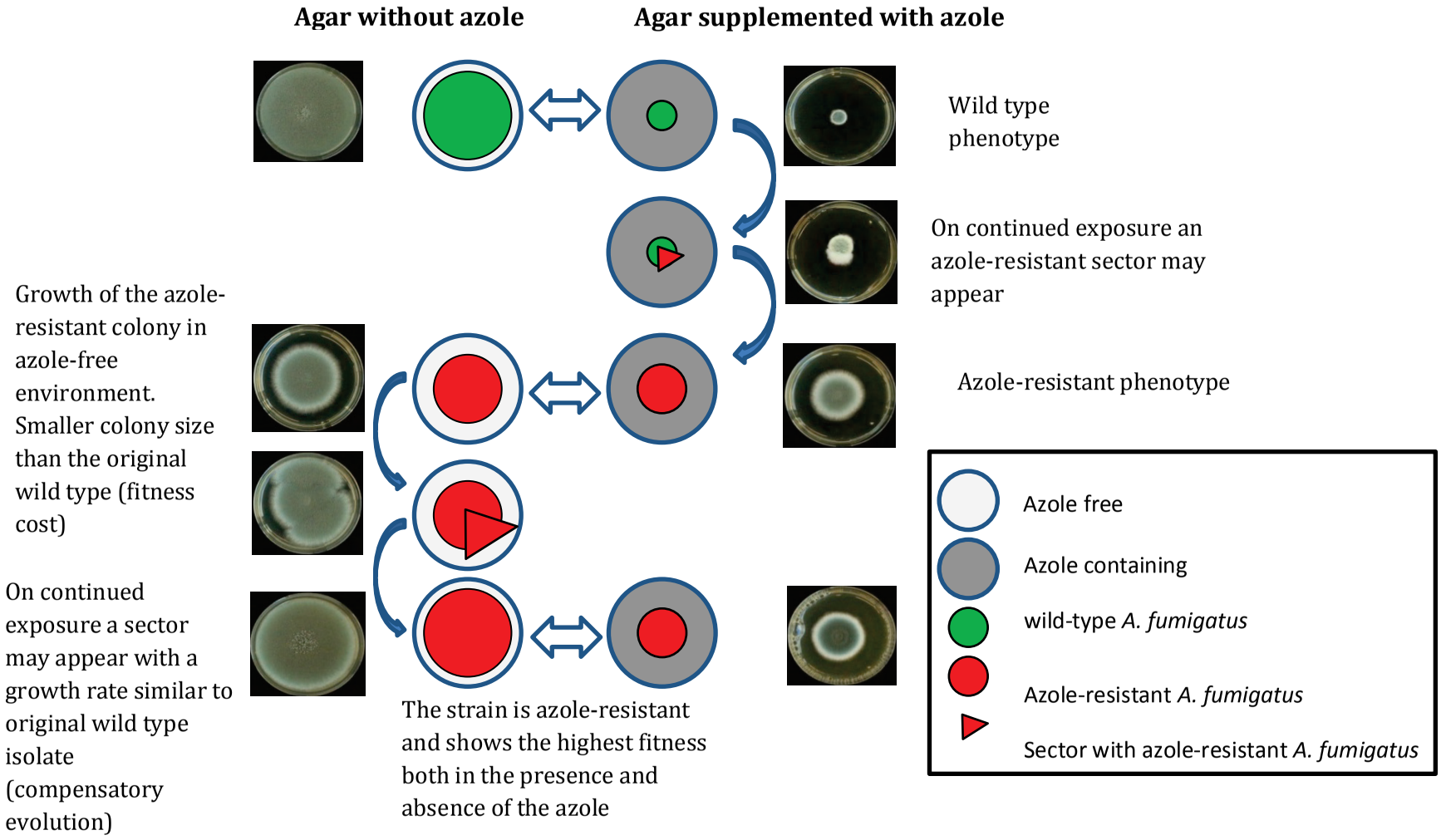

Figure 2. Experimental evolution of *A. fumigatus* in azole-containing and azole-free environments.

Different phases of adaptation are shown of a wild type A. fumigatus during exposure to the azole fungicide difenoconazole at a concentration of 2 mg/l.

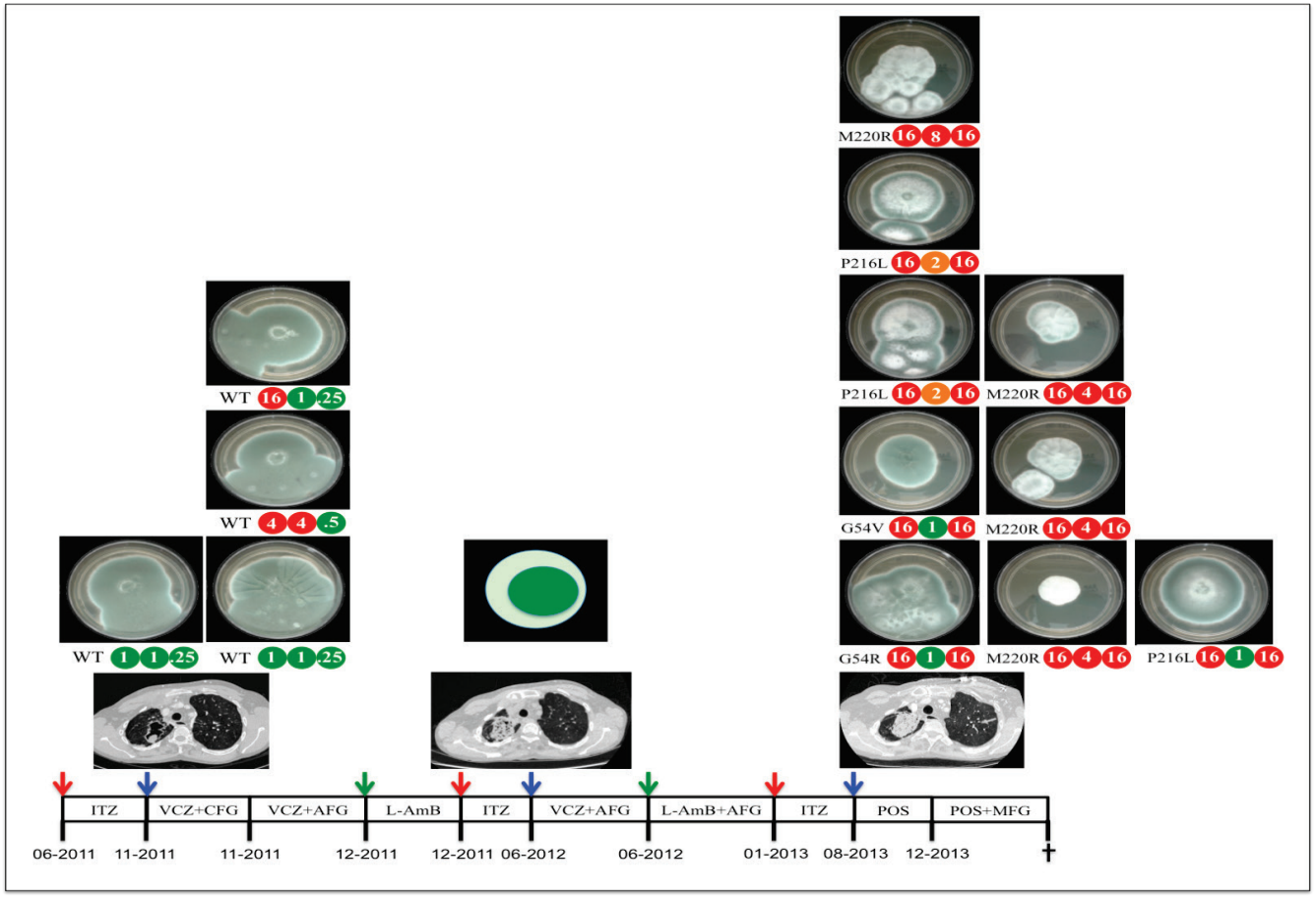

Figure 3. Treatment regimens, evolution of pulmonary aspergilloma and Aspergillus cultures from a CGD patient.

The figure shows consecutive antifungal prophylaxis and treatment regimens between June 2011 and December 2013. The red arrows indicate initiation of azole therapy, the blue arrows indicate switching between azole compounds and the green arrows indicate switching to a polyene-based therapy. The evolution of the pulmonary aspergilloma is shown on CT. A CT was made when the patient was admitted to the hospital for evaluation of respiratory symptoms. The colony morphology of 13 isogenic A. fumigatus isolates is shown on Sabouraud dextrose agar in the absence of azoles. The macroscopic evolution over time is shown together with the azole resistance genotype (Cyp51A mutations) and phenotype: itraconazole, voriconazole and posaconazole MIC (mg/l), respectively. MICs were determined using the EUCAST broth microdilution method. The color of the dots refers to the interpretation with green representing susceptible, orange intermediate and red resistant. In June 2012 A. fumigatus was cultured from sputum, but this culture was not available for analysis.

Abbreviations: ITZ, itraconazole; VCZ, voriconazole; POS, posaconazole; L-AmB, liposomal amphotericin B; CFG, caspofungin; MFG, micafungin. WT, wild type

the patient, but these evolutionary trajectories ultimately benefit the fungus. Our hypothesis is that the longer the fungus is tolerated in the human lung environment and has the opportunity to adapt, the more difficult it will become to achieve eradication. More studies are needed to gain insight in the genomic dynamics of *A. fumigatus* during human infection, as these concepts challenge current management strategies. At present chronic colonization is accepted in some patient groups, especially if the clinical implications of colonization are unclear (CF) or colonization is asymptomatic (aspergilloma). The question is whether this "adaptation opportunity" will decrease the likelihood of fungal eradication when treatment is indicated. Long-term triazole monotherapy is recommended in CPA, with response evaluations after six or nine months of therapy.[65] The accumulation of resistance mutations in triazole-treated patients, indicates that this approach allows for fungal adaptation and questions whether a multi-targeted approach might be more successful in preventing or delaying adaptation. For instance, first-line combination therapy might be more effective, despite the obvious drawbacks of administration of the non-azole drug or antifungal therapy in combination with immune enhancement. For instance, experimental models indicate that the clearance of invasive pulmonary aspergillosis is enhanced by overexpression of gamma interferon.[70]

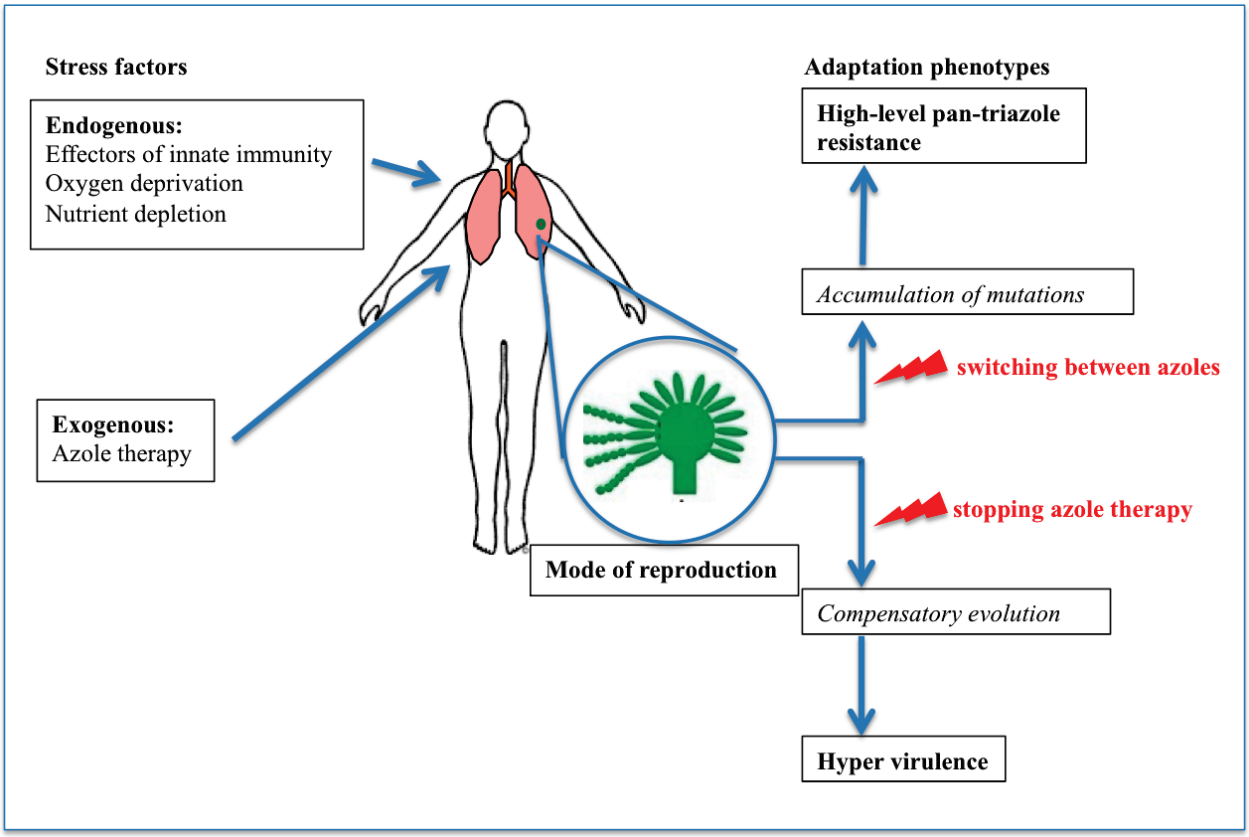

Figure 4. Two scenarios both leading to unfavorable clinical outcomes in patients with persistence of *A. fumigatus* infection

Future research

Adaptation and persistence of *A. fumigatus* will be difficult to avoid as some patients have life-long conditions that make them vulnerable to aspergillosis. However, recent insights in azole-resistance development warrants further research into the biology of *A. fumigatus* in the human environment. It is important to investigate which strategies *A. fumigatus* employs during infection to increase its genetic diversity, especially in relation to the mode of reproduction and *Aspergillus* disease. This starts with analyzing individual colonies cultured from patients with chronic *Aspergillus* diseases. Serially-isolated strains could be subjected to high-resolution genotyping through next-generation sequencing (NGS), as this would give insight in the dynamics of genomic changes. With greater access to this technology studies are emerging that apply NGS to clinical *Aspergillus* isolates, reporting both accumulation of mutations and genomic deletions that appeared to have occurred randomly in isolates recovered from an aspergilloma.[71,72] Genomic changes could also provide clues to the mode of reproduction from which the changes originated. The observed genetic changes need to be correlated to phenotypic changes, such as resistance or general fitness, which might prove difficult given the diversity of stress factors the fungus is exposed to. Therefore, experimental evolution may in addition be utilized to identify specific genes or factors that are associated with adaptation. Furthermore, sexual crossings with *A. fumigatus* have been successfully used to identify mutations that are associated with azole resistance.[73,74] Discovery of such resistance mechanisms allow us to improve (molecular) diagnostics for resistance detection, and may also provide new targets for drug development. With the biologicals that target important components of the anti-*Aspergillus* host defense, such as anti-TNF and anti-IL-17, it can be expected that the inflammatory environment is significantly altered leading to a different composition of immune cells and cytokines and therefore a different environmental pressure on *Aspergillus*. Insights in the fungus-host interaction might provide leads for new treatment strategies and ultimately will help us to improve our management strategies of patients with aspergillosis.

Conclusions

Azole resistance is the showcase of efficient adaptation strategies in *A. fumigatus* in the human host. The fungus can utilize various strategies to overcome environmental stress factors depending on its morphological state. As fungal adaptation results in treatment failure and fungal persistence, the biology of *A. fumigatus* during infection and colonization needs to be understood in order to design strategies that prevent or overcome adaptation.

Search strategy and selection criteria

We searched PubMed from Jan 1, 1966, to March 31, 2016, using the terms "*Aspergillus fumigatus* and azole and resistance" and "fungal and adaptation", and selected the most relevant articles. Only articles in English were reviewed. Relevant references included in these publications were also used. We added additional references from our personal files as needed.

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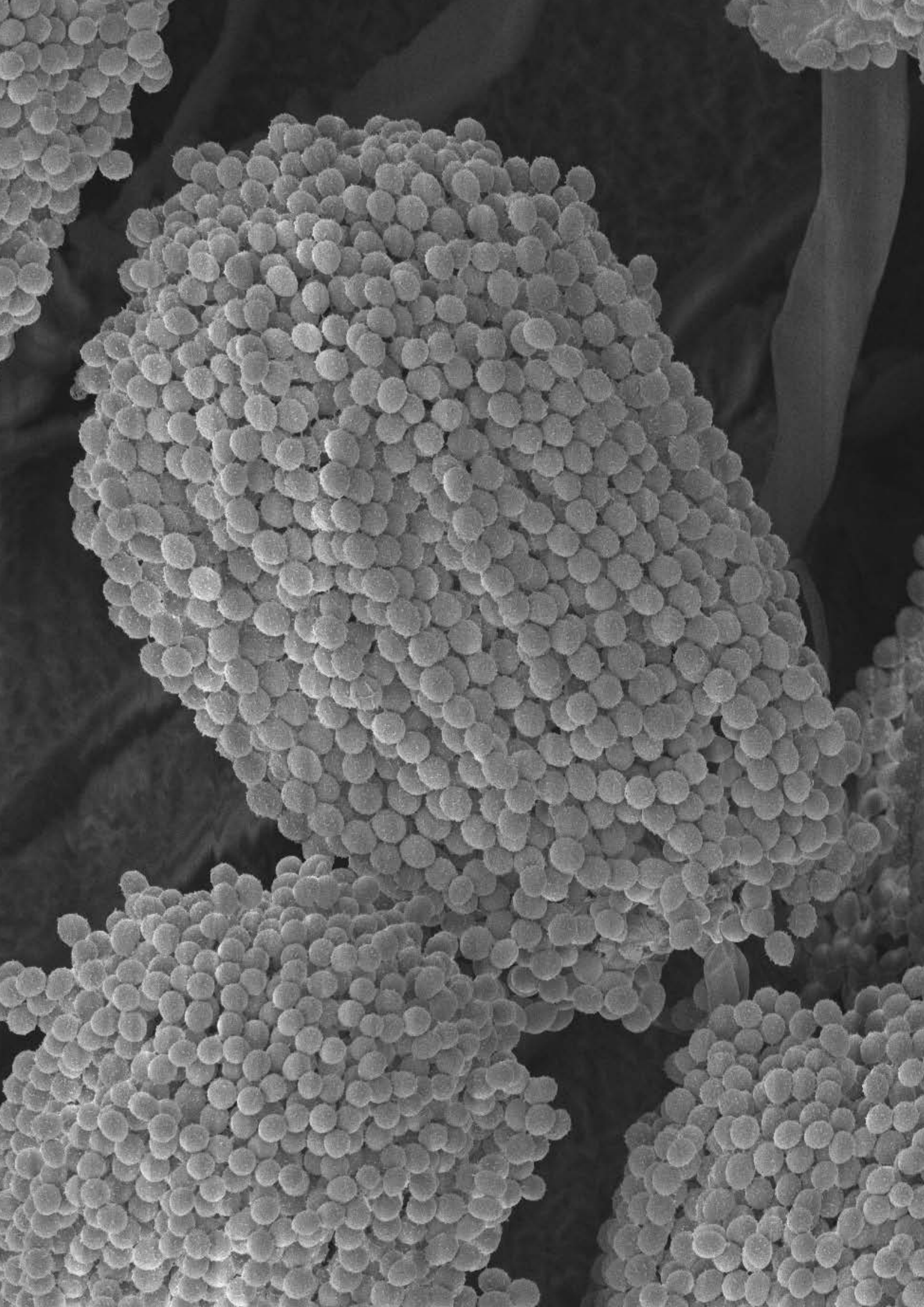

CHAPTER III

Asexual sporulation facilitates adaptation: the emergence of azole resistance in *Aspergillus fumigatus*

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Abstract

Understanding the occurrence and spread of azole resistance in *Aspergillus fumigatus* is crucial for public health. It has been hypothesized that asexual sporulation, which is abundant in nature, is essential for phenotypic expression of azole-resistance mutations in *A. fumigatus* facilitating subsequent spread through natural selection. Furthermore, the disease aspergilloma is associated with asexual sporulation within the lungs of patients and the emergence of azole resistance. This study assessed the evolutionary advantage of asexual sporulation by growing the fungus under pressure of one of five different azole fungicides over seven weeks and by comparing the rate of adaptation between scenarios of culturing with and without asexual sporulation. Results unequivocally show that asexual sporulation facilitates adaptation. This can be explained by the combination of more effective selection because of the transition from a multicellular to a unicellular stage, and by increased mutation supply due to the production of spores, which involves numerous mitotic divisions. Insights from this study are essential to unravel the resistance mechanisms of sporulating pathogens to chemical compounds and disease agents in general, and for designing strategies that prevent or overcome the emerging threat of azole resistance in particular.

Key words: *Aspergillus fumigatus*; azole resistance; asexual sporulation; experimental evolution; alternation between unicellular and multicellular growth; MIC value; mycelial growth rate

Introduction

Aspergillus fumigatus, a common saprophytic fungus, is abundant in soil and decaying organic matter (Bignell 2014; Cuenca-Estrella 2014) and produces numerous airborne spores. As such it is generally considered to be harmless to humans and can be found in houses (e.g. bathrooms), offices (e.g. ventilation vents), and also in hospitals (Haas 2011). However, this fungus may cause a wide range of non-invasive or invasive diseases in immunocompromised patients (Latgé 1999; Rhodes 2006; Snelders et al. 2012; Kwon-Chung and Sugui 2013; Bignell 2014; Rocchi et al. 2014), where invasive aspergillosis is a major cause of mortality and morbidity (Cunha et al. 2013). Azoles are the most widely used antifungal drugs in medical treatment, especially triazoles, such as itraconazole, posaconazole and voriconazole (Albarrag et al. 2011). These triazoles inhibit sterol 14α-demethylase, encoded by the *cyp51A* gene, thereby blocking its function in the fungal ergosterol biosynthesis pathway, resulting in ergosterol depletion and accumulation of toxic sterols (Joseph-Horne and Hollomon 1997).

Unfortunately, the emergence and spread of azole resistance has been increasingly reported, with prevalence from 0.8 - 9.5% in the various medical centres in the Netherlands during 2007-2009 (van der Linden et al. 2011). In the United Kingdom, the National Aspergillosis Centre (Manchester, UK) described an increase of azole-resistant isolates, increasing from 7% in 1997 to 20% in 2009 (Howard et al. 2006; Howard et al. 2009; Bueid et al. 2010). Indeed, azole resistance has been reported also in other European countries, the Middle East, Asia, and most recently in Africa (Snelders et al. 2008; Arendrup et al. 2010; Lockhart et al. 2011; Mortensen et al. 2011; Pfaller et al. 2011; Chowdhary et al. 2012; Morio et al. 2012; Gisi 2013; Bignell 2014; Chowdhary et al. 2014). Clearly, azole resistance is a growing concern as patients with azole-resistant *A. fumigatus* have a high probability of treatment failure, and alternative treatment options are limited (Seyedmousavi et al. 2014). To control this problem we need to elucidate by what mechanism(s) the resistance emerges, how it can spread, and how resistant genotypes can persist in environments without azoles.

Several studies focussed on the origin of azole resistance and tested *A. fumigatus* strains for azole resistance isolated from both clinical and environmental settings (Mellado et al. 2007; Howard et al. 2009; Snelders et al. 2009; Arendrup et al. 2010; Mortensen et al. 2010). These studies showed that highly resistant strains were recovered from compost, flowerbeds and agricultural fields, as well as from patients with *Aspergillus* diseases. In addition, recent studies indicated that azoles used in agricultural settings can select for resistance (Snelders et al. 2012; Chowdhary et al. 2013; Verweij et al. 2013; Bowyer and Denning 2014), especially the azole fungicides bromuconazole, tebuconazole, epoxiconazole, difenoconazole and propiconazole, which are widely used in crop protection and material preservation. It is hypothesised that resistance to these fungicides is selected especially in these non-clinical environments. When susceptible patients subsequently inhale fungicide-resistant *A. fumigatus* spores, *Aspergillus* disease

may develop to which the medical triazoles are ineffective due to high molecule similarity to the five "environmental" azole fungicides. However, previous studies did not allow for the identification of the factors facilitating the emergence of resistance and the responsible evolutionary mechanism(s). This is what we address in the present study.

This study focuses on the asexual stages of the life cycle of *A. fumigatus* and in particular on the possible role of asexual sporulation in facilitating adaptation of the fungus to azole environments. Similar to that of the model species *A. nidulans*, unicellular and multicellular states alternate during the life cycle (whether it be asexual, sexual, or parasexual) and selection can act on both states (Casselton and Zolan 2002; Bruggeman et al. 2004; Mah and Yu 2006; Schoustra et al. 2006; O'Gorman et al. 2008). The asexual part of the life cycle is by far the most common in nature providing an alternation between multicellular and unicellular states by the generation of ubiquitous uninucleate asexual spores (through numerous mitotic divisions) that after dispersal and germination can generate multinucleate mycelia that again produce numerous spores that become airborne (Figure 1A).

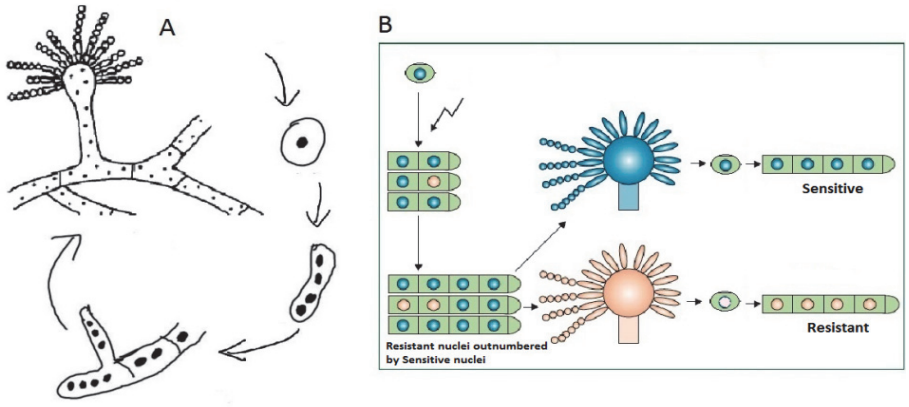

Figure 1. The asexual life cycle of *Aspergillus fumigatus* and hypothesis of escaping strategy

A: The asexual cycle starts with the formation of a mycelium that can be initiated by a single uninucleate haploid spore (either an asexual conidiospore or a sexual ascospore). Mitotic division in the mycelium takes place in the apical cells at the growing front of the colony (Casselton, 2002). After one to two days, mycelial cells differentiate into aerial hyphae with a foot cell and a spore head (conidiophore) covered with conidiogenous cells (phialides). Around three days after initiation of the multicellular mycelium these conidiogenous cells produce numerous unicellular and uninucleate asexual spores by mitotic divisions. Thus a single spore may give rise to up to 10^9 new asexual spores in 3-4 days under favourable conditions.

B: In the multicellular mycelium with mixed nuclei, resistant mutated nuclei have limited or no effect on the resistance phenotype as they are outnumbered by sensitive nuclei. After asexual sporulation (alternation from multicellular mycelium to unicellular spores), the resistant and sensitive nuclei get separated and the former can express their superior phenotypes in an azole environment (modified with permission from P. E Verweij, (Verweij, 2009).

Previously, Verweij *et al.* hypothesized that asexual sporulation is essential for phenotypic expression of azole-resistance mutations in *A. fumigatus* in clinical settings

(Verweij et al. 2009). This idea is based on the fact that when a resistance mutation occurs during vegetative growth in the multinucleate mycelium, the resulting resistant nucleus is initially surrounded by sensitive nuclei in a heterokaryotic cell. All else being equal, if resistance mutations are at least partially recessive, resistance is not fully expressed into the phenotype when also sensitive nuclei are present in the mycelium. Crucially then, the formation of uninucleate asexual spores would allow resistant nuclei to escape from the heterokaryotic mycelium so that, after germination, the resistant phenotype would be fully expressed (Figure 1B). This mechanism could take place within the lungs of patients, since sporulation propagative structures have been observed in patients with cavitary *Aspergillus* diseases. Furthermore, resistance selection during azole therapy has been reported in these patients (Camps et al. 2012). In cavitary *Aspergillus* diseases, such as chronic cavitary aspergillosis and aspergilloma, *A. fumigatus* undergoes asexual sporulation, as opposed to invasive aspergillosis, which involves only hyphal growth. This clinical observation also shows that various fungal morphotypes exist within patients and suggests that there is a link between sporulation and resistance development in patients. Therefore, it is crucial to study the role and consequence of asexual sporulation to understand the invasive infection in immune-compromised patients.

In this study, we investigated the effect of asexual sporulation on the emergence of resistance to five different azole fungicides of *A. fumigatus* in a laboratory evolutionary experiment. This was done by contrasting the rate of development of resistance to azoles in *A. fumigatus* during seven weeks, in six replicate cultures, with two different treatments: cultured either without asexual sporulation (continued mycelium growth) or serially cultured allowing asexual sporulation. For the experimentally evolved cultures of each contrast and replicate, we measured resistance levels and mycelial growth rate (fitness). Five different azole fungicides were compared for their induced levels of azole resistance. Furthermore, we assayed whether resistance mutations were dominant or recessive in one of the evolutionary lineages, testing the underlying assumption that resistant nuclei are not (fully) expressed in the phenotype of multicellular mycelium.

Materials and Methods

Fungal isolates and azole fungicides

Azole fungicides (bromuconazole [b], tebuconazole [t], epoxiconazole [e], difenoconazole [d], and propiconazole [p]) were purchased from Sigma Company (Sigma Aldrich, Germany). *Aspergillus fumigatus* CBS 140053 was isolated from an environmental field in Wageningen in 1992 in our laboratory at Wageningen University before these azole fungicides were applied to the environment (Snelders et al. 2012;

Bowyer and Denning 2014). This strain is sensitive to our five azole fungicides and was used as the ancestor of the evolution experiment.

Experimental evolution of *A. fumigatus* with and without asexual sporulation under the selection pressure of azole fungicides

In a seven-week experimental evolution set-up, the culturing of *A. fumigatus* CBS 140053 with and without asexual sporulation was contrasted. For both treatments we founded six lineages for each of the five azole fungicides and six lineages without any fungicide as a control. Seven time points for each lineage were sampled and stored in our "frozen fossil record". Thus, the total number of samples was 505 [two treatments * (5 azoles + control) * 6 replicates * 7 time points + ancestor = 505].

For the serial sub-culturing treatment allowing for asexual sporulation, parallel lineages were inoculated with 10^4 ancestral spores in a 5 µl droplet into a bottle with 10 ml solid Malt Extract Agar (MEA) medium, which contained 1 µg/mL of one of the five azole fungicides. This amount of azole is based on the application of these fungicides estimated if a soil layer of 1 cm is considered in agricultural fields (Gisi 2013). Under this concentration *A. fumigatus* is able to sporulate. After seven days of incubation at 37 °C, all material including spores from each bottle was harvested: 2 mL of saline (distilled water with NaCl 0.8 g/L) supplemented with Tween 80 (0.05 % v/v) and beads were added to the bottle that was subsequently vortexed for 5 minutes (Sigma Aldrich, Germany). Five µL of each suspension was used to initiate the next selection cycle and the rest was stored at -80°C after adding one volume of a 50% glycerol solution for the "frozen fossil record"(Schoustra et al. 2006; Schoustra et al. 2009). This procedure was repeated every seven days for seven rounds of selection.

For the treatment where asexual sporulation was not allowed, cultures were initiated with spores at one end of a race tube and allowed to grow undisturbed for seven weeks. A race tube is a 50 mL disposable pipette filled with 25 mL MEA medium, and for the azole treatment supplemented with 1 µg/mL of one of each of the azole fungicides, solidified horizontally, in which the mycelial growth can be tracked. The race tubes were then incubated in a 37 °C incubator for seven weeks. Without disturbing the race tubes (so that the asexual spores produced behind the growth front of the mycelia could not contribute to the development of azole resistance), the growth-front of the fungi was marked every week. After seven weeks of continued mycelial growth, the race tubes were opened at the weekly markings to harvest material that we suspended in saline (distilled water with NaCl 0.8 g/L) supplemented with Tween 80 (0.05% v/v) and one volume of a 50% glycerol solution for storage in our "frozen fossil record".

Resistance testing of cultures evolved with and without asexual sporulation against azole fungicides

The resistance level of all lineages that had evolved in the presence or absence of azole fungicides over seven weeks was tested. Half the cultures had gone through seven weekly cycles of asexual sporulation, while the other half had grown by seven weeks of mycelial extension continuously. Resistance level was defined as the minimal inhibitory concentration (MIC) of the azole fungicide that results in 100% growth inhibition of fungal growth. The relative MIC is defined as the average MIC divided by the MIC of the azole fungicide sensitive ancestor *A. fumigatus* CBS 140053. The resistance test was performed following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) reference method (Lass-Flörl et al. 2008): RPMI-1640- 2 % glucose medium (Sigma Aldrich, Germany) was supplemented with bromuconazole, tebuconazole, epoxiconazole, difenoconazole or propiconazole, ranging from 0.016 to 16 mg/L in two-fold concentration steps and loaded into 96-well plates. Spore suspensions used to initiate the assay were prepared in saline with 0.05% Tween 80 (Sigma Aldrich, Germany) and counted on a Coulter counter (Coulter USA) to standardize the concentration of spores to 1-2.5×10^5 CFU/mL (Bos et al. 1988). The standardized spore suspension was inoculated into flat-bottomed 96-well microtiter plates containing a series with increasing amounts of fungicide in RPMI-1640- 2% G medium, and incubated at 37°C for 48 h. Readings from the bottom of the microtiter plates were performed using a mirror to determine at which concentration we observed 100 % inhibition, resulting in the MIC value estimates (Camps et al. 2012). Six replicates for each assay were used. We (arbitrarily) classified the MIC as 32 mg/L when we observed no inhibition at the concentration of 16 mg/L.

Mycelial growth rate of cultures evolved with and without asexual sporulation

The mycelial growth rate (MGR) of all cultures that had evolved under laboratory conditions in the presence or absence of azole fungicides over seven weeks was assayed. Spore suspensions of all evolved cultures were inoculated onto Petri dishes with solid MEA medium containing 1 µg/mL of the azole fungicide that was used during the evolution experiment, either with an inoculation needle or with a five µL spore suspension. After 4 days of incubation at 37 °C, the MGR was determined by averaging the colony diameters (in mm) as measured in two randomly chosen perpendicular directions. The relative MGR is defined as the MGR divided by the colony diameter of the azole fungicides sensitive ancestor *A. fumigatus* CBS 140053, grown on MEA medium without azole fungicides (De Visser et al. 1997; Schoustra et al. 2006; Schoustra et al. 2009).

Dominance test of resistance mutation in diploids and heterokaryon of one of the evolutionary lineages

A. fumigatus predominantly grows as a haploid. Whether azole-resistance mutations were dominant or recessive in a heterozygous diploid as well as in a heterokaryon (mycelium containing multiple types of nuclei) was investigated from one of the evolutionary lineages. Spontaneous nitrate non-utilizing mutations *nia* (nitrate non-utilizing) and *cnx* (nitrate and hypoxanthine non-utilizing) were selected on the basis of chlorate resistance as selective complementing markers to facilitate heterokaryon and diploid construction (Cove 1976; Debets et al. 1990). Heterokaryons and diploids were constructed using standard methods: an azole sensitive *nia*-strain and a highly azole resistant *cnx* –strain (D1-7#, derived from the end culture evolved in the presence of difenoconazole) were used for selection of heterokaryons on minimal medium (MM) with nitrate as the only N-source. Asexual spores from heterokaryons were harvested and plated in sandwich plates to select for heterozygous diploids (Todd et al. 2007). We found a frequency of heterozygous diploid spores of approximately 10^{-5}. Diploidy was verified by measuring the size of condiospores - the diameter of spores of diploids is 3.2 µm (±0.2 SEM), which is 1.45 times that of haploids (2.2 µm ±0.2SEM) - the volume of diploid spores thus being three times that of haploids. The growth of the azole sensitive *nia* mutant and the highly azole-resistant evolved strain D1-7# (cnx), were tested on MM+5mM urea with 1 µg/ml concentration of difenoconazole. The heterokaryon and diploid were tested on MM medium with 1µg/ml concentration of difenoconazole. The level of resistance was measured as the relative MGR (see above).

Stability test of azole fungicides over the evolutionary experiment

In order to verify whether the five azole fungicides were stable during the seven weeks of experimental evolution, 24 petri dishes with 1 µg/mL of each of the five azole fungicides were prepared. Of these, 21 plates were incubated at 37 °C and the remaining three plates were kept at 4 °C as the control. At the end of each week, three plates were removed from the incubator and stored at 4 °C till the end of week seven, when all plates had been removed. After this, 5 µL of a fresh ancestor *A. fumigatus* CBS 140053 spore suspension was inoculated in the middle of each plate and incubated at 37 °C for five days. The mycelial growth rate on all plates was recorded, MGR was compared to the three stored control plates and the known MGR of the strain on freshly prepared plates.

Statistical analysis

We used a general linear model (analysis of variance; ANOVA) that included the fixed factors “sporulation treatment” and “azole fungicide”, and their interaction, to explain the observed variation in relative MGR and relative MIC of the evolved strains (model: Relative MGR~ azole fungicide (A), sporulation treatment (S), A*S; Relative MIC~ azole fungicide (A), sporulation treatment (S), A*S). Furthermore, since the effects of the sporulation treatment depended on the azole fungicide (see Results), we performed one

way ANOVAs to test for the effect of sporulation treatment for each azole separately (model for each azole separately: Relative MGR~ sporulation treatment and Relative MIC~ sporulation treatment). Finally, a one way ANOVA and following post-hoc LSD tests were used to check whether the achieved level of resistance depended on the azole fungicide used for selection. This was done separately for each sporulation treatment, using the following model (Relative MGR~ azole treatment, and Relative MIC~ azole treatment). A one way ANOVA and following post-hoc LSD tests were also used to test the difference in the induced level among five azole fungicides, for the difference in the MGR of diploid, haploid and heterokaryons.

Results

Asexual sporulation enhanced resistance level over evolutionary time

The resistance level was assayed of all lineages that had evolved under laboratory conditions in the presence or absence of an azole fungicide over seven weeks. Half the cultures had gone through seven cycles of asexual sporulation, while the other half had grown continuously by mycelial extension. Five different azole fungicides were applied. Figures 2A and 2B show the average resistance level (defined as the relative Minimal Inhibitory Concentration, of a given azole relative to the MIC of the ancestor) and evolutionary dynamics of the average of the six replicate lineages within each treatment. Our statistical analysis showed that the sporulation treatment is a key factor ($F_{1, 50}$ = 31.696, P < 0.0001; Supplement 1A) in explaining the differences in the relative MIC between evolved strains and that these differences also depended on which azole fungicide was used (Sporulation treatment*Azole fungicide interaction, $F_{4, 50}$=2.674, P < 0.05; Supplement 1A). When testing for the effect of the sporulation treatment for each azole separately, we found that for bromuconazole and difenoconazole, the relative MIC of cultures that had evolved with sporulation was significantly higher than the relative MIC of cultures without sporulation (for bromuconazole $F_{1, 10}$ = 8.448; P < 0.05; for difenoconazole $F_{1, 10}$ = 22.727, P < 0.01; Supplement 1B).

For the cultures that evolved in the presence of fungicide and with asexual sporulation, we found that the relative MIC values significantly increased at the end of our experiment compared with the treatment without azoles (control treatment) (ANOVA, $F_{5, 30}$ = 4.854; P < 0.05; Post Hoc LSD Test, P < 0.05, except propiconazole P > 0.05; Supplement 1C). The relative MIC values started to increase right at the first cycle and levelled off around the sixth week (Figure 2A). The overall average increase in relative MIC over all fungicides was 3.67±0.90 SEM, with the increase depending on which azole was used for resistance selection (Table 1).

For the cultures that evolved without asexual sporulation, exposure to azole fungicides also increased the relative MIC values compared to control lineages growing in the

absence of azole fungicides (ANOVA, $F_{5, 30} = 8.417$ $P < 0.001$; Post Hoc LSD Test $P < 0.01$ except for difenoconazole and epoxiconazole $P > 0.05$; Figure 2B, Supplement 1D). The relative MIC value started to increase at the first week and then all remained stable during the following six weeks. The average increase in relative MIC over all fungicides was 1.86 ± 0.56 SEM, with again the increase depending on which azole was used for resistance selection (Table 1). Crucially, although the azole-resistance levels also increased in the treatment without sporulation, the average relative MIC value for cultures with asexual sporulation was twice as high as that of the cultures without asexual sporulation.

When analyzing the absolute MIC values of the evolved strains, we reach the same conclusion that asexual sporulation results in higher azole-resistance levels; data and related discussion are in Supplement 2.

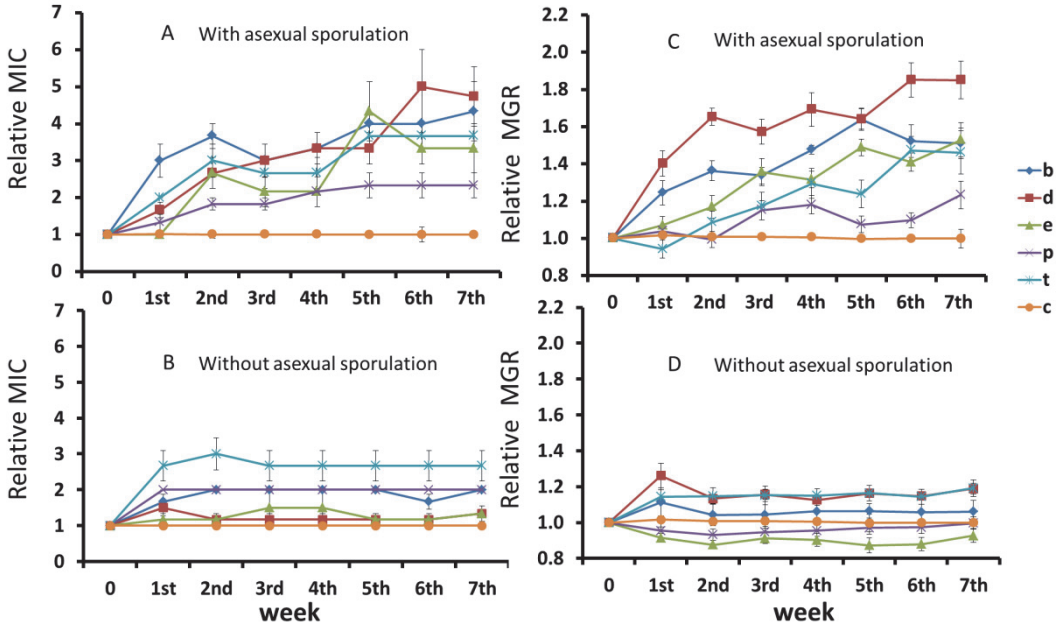

Figure 2. Relative MIC value (Minimal Inhibitory Concentration corresponding to the level of resistance) and relative Mycelial Growth Rate (MGR) of azole fungicides for 504 evolved cultures with and without asexual sporulation compared to the ancestor in the presence of one of five azole fungicides (concentration: 1µg /mL)

(b=bromuconazole, t=tebuconazole, e=epoxiconazole, d= difenoconazole and p=propiconazole, c=control: no fungicide. Lines show the average of six parallel lineages for each treatment). Error bars indicate the standard error of the mean (SEM).

A,B: The relative MIC of cultures with and without asexual sporulation

C,D: The relative MGR of cultures with and without asexual sporulation

Asexual sporulation increased mycelial growth rate (MGR) and correlates with resistance level increase

Relative Mycelial Growth Rate (MGR), as a reliable measure for overall fitness in filamentous fungi, was assayed for all cultures that had evolved under laboratory conditions in the presence or absence of azole fungicides over seven weeks.

Figures 2C and 2D show the MGR changes and evolutionary dynamics averaged over the six replicate lineages within each treatment. Statistical analysis revealed that asexual sporulation and azole type both are significant factors for explaining the differences in relative MGR and MIC (sporulation treatment: $F_{1, 50} = 94.402$; $P < 0.001$; azole treatment: $F_{4, 50}=8.471$, $P < 0.001$). Similar to our analysis for relative MIC, there is a significant interaction between the two factors ($F_{4, 50} = 3.509$, $P < 0.05$; supplement 1E). In the model testing the effect of sporulation treatment for each azole separately, the relative MGR of cultures evolved with asexual sporulation is significantly higher than the relative MGR of cultures without asexual sporulation (one way ANOVA, all P <0.01, except tebuconazole; Supplement 1F).

For the cultures that were allowed asexual sporulation, it was found that the relative MGR significantly increased at the end of the seven weeks (Figure 2C) compared to the control (ANOVA, $F_{5, 30} = 11.267$, $P < 0.001$; Post Hoc LSD Test all $P< 0.01$, except propiconazole $P > 0.05$; Supplement 1G). The overall average increase in relative MGR over all fungicides was 1.52 ± 0.05. The relative MGR started to increase at the first cycle and appeared to level off around the sixth cycle. As reported for the MIC data, the increase in relative MGR depended on which azole was used for resistance selection (Table 1).

For the relative MGR of cultures that evolved without asexual sporulation, it was found that the relative MGR significantly changed over the course of the experiment (ANOVA, $F_{5, 30} = 9.411$, $P < 0.001$; Post Hoc LSD Test showed a significant effect of difenoconazole and tebuconazole $P < 0.01$; Figure 2D, Supplement 1H). The overall average increase in relative MGR over all fungicides was 1.08 ± 0.03SEM. The relative MGR increase started at the first cycle, but already levelled off after the first cycle. Similar to the results and analyses for relative MIC, relative MGR values after seven weeks for cultures with asexual sporulation were much higher than those of the cultures without sporulation.

Comparing the evolutionary changes for the five different azole fungicides used within the asexual sporulation treatment, we observed that the selection response differs for the different azole fungicides (for relative MGR ANOVA, $F_{4, 25} = 5.469$, $P < 0.01$ and relative MIC showed similar results; post-hoc LSD pairwise comparisons between all five azole fungicides are in Supplement 1I). Difenoconazole imposes the strongest selection as shown by the highest increase in relative MGR and MIC whereas this pressure was much less strong for propiconazole (Figure 3). Another possible explanation is that the mutation rate is higher in the presence of difenoconazole than the other drugs.

Generally, relative MIC is highly and significantly correlated with the relative MGR (linear regression over all treatments, r = 0.811, $F_{1, 38}$ =72.918, P < 0.01) over all cultures within the asexual sporulation treatment, and also for each of the azole fungicides separately, except for propiconazole (Table 1). This indicates that the MGR change of cultures was due to the change in resistance level. In other words, the change in MIC is reflected in a change in MGR. This is in agreement with previous studies, which showed that colony diameter (mycelial growth rate) is a good and reliable measure of overall fitness (assuming MIC itself is an important fitness component), since it is highly

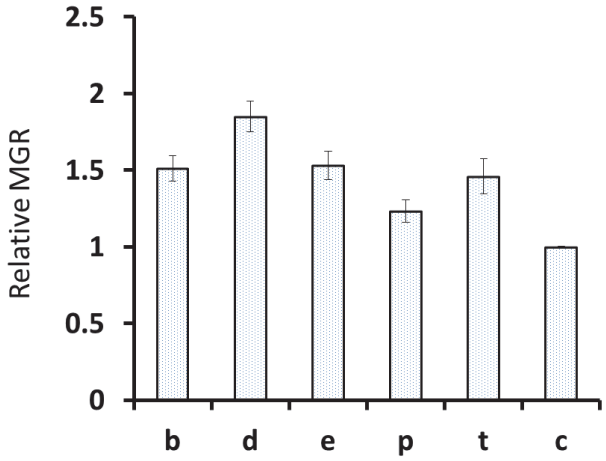

Figure 3. Relative MGR values of azole fungicides for evolved cultures with asexual sporulation in the presence of one of five different azole fungicides

(b=bromuconazole, t=tebuconazole, e=epoxiconazole, d= difenoconazole and p=propiconazole, c=control, no fungicide). Bars show averages of 6 replicate lineages. Error bars show indicate the standard error of the mean (SEM).

correlated with spore production (De Visser et al. 1997; Pringle and Taylor 2002; Schoustra et al. 2009; Schoustra and Punzalan 2012). When analyzing the absolute MGR values of the evolved strains, we reach the same conclusion that asexual sporulation results in higher azole resistance levels; data and related discussion are in Supplement 2.

To verify that increases in both MIC and MGR were due to the actual fixation of mutations, a sexual cross was performed between the ancestral genotype and an evolved strain with high MIC and MGR. The results show a clear Mendelian segregation of fitness among the progeny, confirming that fitness increase is due to the fixation of mutations (Supplement 3).

Stability test of azoles fungicides over the evolutionary experiment

The stability of the five azole fungicides during experimental evolution was tested by growing the ancestor on media with azoles that was first stored for up to seven weeks at 37 °C. No significant differences in relative MGR were found between fungus inoculated on these pre-incubated media and those on the freshly prepared media (ANOVA, $F_{7,120}$ = 0.442, P = 0.874; Supplement 4). This indicates that all five azole fungicides were stable during the seven weeks of our experimental evolution.

Table 1.The relative MIC and MGR of evolved cultures with and without asexual sporulation and correlation between relative MIC and MGR in the cultures with asexual sporulation.

Azole used during experimental evolution	Endpoint cultures evolved with asexual sporulation			Endpoint cultures evolved without asexual sporulation	
	Relative MIC(azoles) Av. ± SEM.*	Relative MGR(azoles) Av. ± SEM	Correlation between MIC and MGR (r^2, p)	Relative MIC(azoles) Av. ± SEM	Relative MGR(azoles) Av. ± SEM
Bromuconazole(b)	4.33 ± 1.97	1.51 ± 0.20	r^2 =0.845, P=0.001	2.00 ± 0.00	1.06 ± 0.14
Difenoconazole(d)	4.63 ± 1.63	1.85 ± 0.24	r^2 = 0.855, P=0.001	1.33 ± 0.52	1.19 ± 0.08
Epoxiconazole(e)	3.33 ± 2.42	1.53 ± 0.23	r^2 =0.758, P=0.005	1.33 ± 0.52	0.93 ± 0.09
Propiconazole(p)	2.33 ± 0.82	1.23 ± 0.18	r^2 =0.411, P = 0.087	2.00 ± 0.00	0.99 ± 0.07
Tebuconazole(t)	3.67 ± 0.81	1.46 ± 0.33	r^2 = 0.629, P=0.019	2.67 ± 1.03	1.19 ± 0.12
No azole (control)	1.00 ± 0.00	0.98 ± 0.05	r^2 = 0.99, P<0.001	1.00 ± 0.00	1.00 ± 0.00

*: six biological replicates

Incomplete dominance of resistance mutation in diploids and heterokaryons

A. fumigatus normally grows as a vegetative haploid, although vegetative diploids also exist at very low frequency. The dominance level of azole-resistance mutations in one of the evolutionary lineages was assayed in heterozygous diploids as well as in heterokaryons. The relative MGR of the haploid sensitive strain, the highly resistant haploid evolved strain, the heterokaryon, and the heterozygous diploid were assayed on medium with 1 µg/mL of difenoconazole (Figure 4). The relative MGR values (± SEM over 3 replicates) of sensitive *A. fumigatus* CBS 140053, resistant D1-7, diploid and heterokaryon were respectively 0.17± 0.007; 0.43 ± 0.012; 0.33 ±0.0024; 0.36 ±0.0027. The relative MGR differed significantly among the different strains (ANOVA $F_{3, 8}$=3749.663, P < 0.001; Post Hoc LSD Test: all pair-wise contrasts: P <0.001). The growth of the heterozygous diploid was 78.5 % of that of the resistant strain, while the growth of the heterokaryon was 83.7 % of the resistant strain. Both the phenotype of the diploid and the heterokaryon indicated that the mutation is incomplete dominant.

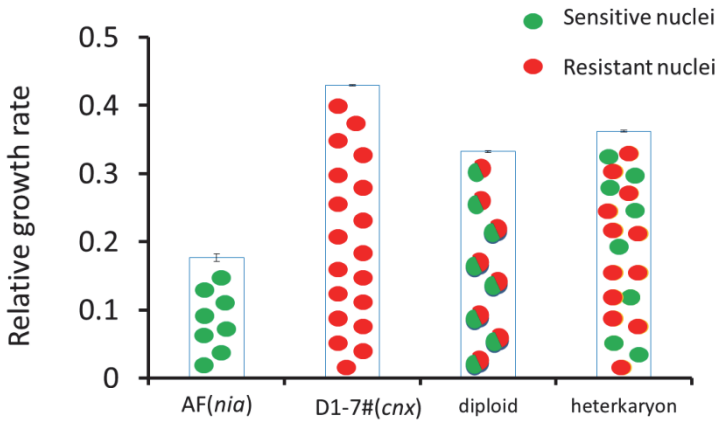

Figure 4. Relative mycelial growth rate (relative MGR) of the heterokaryon, the heterozygous diploid, the sensitive haploid (AF *nia*), and the resistant haploid strain (*cnx* D1-7#).

The relative MGR is defined as the MGR of the strains grown on MEA medium with azole fungicides divided by the colony diameter of the strains grown on MEA medium without azole fungicides. Error bars indicate standard error of the mean (SEM). All strains have a significantly different relative growth rate at the P<0.001 level.

Discussion

Asexual sporulation results in rapid evolution of high resistance to azole fungicides in *A. fumigatus*

We evaluated whether asexual sporulation promotes adaptation to an azole-fungicide environment in *A. fumigatus*, i.e. the development of resistance. We contrasted the development of resistance to five different azole fungicides between populations that went through repeated rounds of asexual sporulation with continued multicellular growth without asexual sporulation. We found clear differences in the achieved levels of resistance between cultures that had evolved with or without asexual sporulation, sporulation leading to roughly twice as high resistance levels. Generally, the relative MIC correlated well with relative MGR for all cultures. Evolutionary trajectories of cultures with asexual sporulation showed multiple steps of increase in both relative MIC and MGR, which may be indicative of the successive fixation of multiple mutations (Lenski and Travisano 1994; Schoustra et al. 2009), although exceptions have recently been reported (Lang et al. 2013). In contrast, the resistance level of the cultures growing without asexual sporulation did not further increase after taking a first (mutational) step.

Evolutionary mechanisms underpinning the emergence of azole resistance

Our results show that the resistance level to azoles gradually increases when the culture goes through asexual sporulation, which suggests that asexual sporulation contributes to, and promotes, the evolution of resistance. This may be explained either by more efficient selection for resistance and/or a higher mutation supply in the sporulating cultures.

Asexual sporulation in *A. fumigatus* enables the phenotypic expression of the resistance by providing an alternation between multicellular and unicellular stages. This alternation provides the opportunity for mutations to be released from the multicellular mycelium and to fully express its phenotype into a newly formed mycelium founded by that spore. Life cycles with both multicellular and unicellular phases are widespread in animals, plants, and some fungi (Maynard Smith 1988; Anderson 1992; Kondrashov 1994b; Michod 2007). Grosberg and Strathmann (1998) argued that the multicellular stage reduces genetic variability and leads to inefficient selection compared to the unicellular stage(Grosberg and Strathmann 1998). For instance, the unicellular stage in the life cycle of multicellular organisms allows for the purging of deleterious mutations by exposing the individual gametes as well as the resulting zygote to selection, while at the same time this unicellular stage reduces conflicts of interest among genetically different replicators (e.g. mitochondria) within a multicellular organism (Grosberg and Strathmann 1998; Bastiaans et al. 2014). Moreover, the alternation of multicellular and unicellular stages also promotes the selection of beneficial mutations. In the case of *A. fumigatus*, this alternation includes a uni-nucleate spore stage after which a new multicellular mycelium will form. This will reduce variability in the total number of mutations per individual by either removing unique mutations or by completely fixing them in a culture.

Following this logic, we can argue that the asexual sporulation process released the mutations from the multicellular mycelium to allow for efficient selection and expression of beneficial traits. Blocking sporulation could thus reduce evolvability (Kondrashov 1994b, a). This aligns with the hypothesis that asexual sporulation is essential for phenotypic expression of azole resistance mutations in *A. fumigatus*. During continued mycelium growth, individual resistant nuclei may arise but these are outnumbered by sensitive nuclei. The resistant nuclei can only escape through asexual sporulation after which they end up in unicellular resistant spores that may then give rise to a resistant mycelium. Thus exclusive mycelial growth (without asexual sporulation) prevents the emergence of resistance in *A. fumigatus*, at least over timescales that are relevant for the treatment of patients.

The mechanism of release of resistant nuclei from a sensitive mycelium will work most efficiently when the azole resistance mutation is recessive and therefore needs homokaryon formation for the full expression of the resistance. Therefore, we assayed whether azole-resistance mutations from one of the evolutionary lineages are dominant

or recessive. We constructed heterozygous diploids and heterokaryons and compared the resistance level with the haploid sensitive and resistant strains. Both the phenotype of diploid and heterokaryon indicated that the mutation showed incomplete dominance. Thus homokaryon formation by asexual sporulation will result in the full expression of the azole-resistant genotype, and will hence enhance the spread of the mutation(s). The extent of this benefit for resistance mutations will be dependent on the selection pressure imposed by the (azole) environment. When high concentrations of azoles are used, selection will be stronger on the resistant phenotype, and thus the benefit to a full expression of the phenotype will be higher. The significance of the contribution of the sporulation process to the emergence of azole resistance is thus expected to depend on the precise application of the azoles in clinical and agricultural settings.

Furthermore, in terms of spores contributing to azole-resistance development, asexual sporulation greatly increases the effective population size allowing for many more possible mutants to be exposed to selection and also increases the mutation supply through the additional mitoses required to generate all the spores. Typically after one week of growth, a colony of *A. fumigatus* may contain up to 10^9 spores (Cole 1986; Latgé 1999; Dagenais and Keller 2009; Amorim et al. 2010; Gifford and Schoustra 2013; Gisi 2013). While the number of nuclei needed to populate the mycelium is limited, upon sporulation, the formation of hundreds of millions of spores on top of the mycelium requires additional mitotic divisions, with the possibility of mutations arising during DNA replication. Therefore, the total number of nuclei produced by mitotic divisions during sporulation by far exceeds that present in the mycelium. As a result, *de novo* resistance mutations are more likely to occur during asexual sporulation than in the expanding mycelium. The occurrence of mutations has been demonstrated in *Aspergillus* and yeast during both meiosis and mitosis (Magni and Von Borstel 1962; Holliday 1964; Käfer 1977; Esposito et al. 1982). Thus a large spore production is likely to generate numerous unique genotypes carrying mutations that can be tested by natural selection, for instance in environments with azoles. In addition, resistance mutations are more likely to be selected from asexual sporulation due to the single-celled nature of the spores that removes the burden of the (partial) recessivity of the mutations that shields the full expression of resistance in a multicellular mycelium.

Other potential resistance mechanisms

Aneuploidy is well known to underlie azole resistance in both *Candida* and *Cryptococcus*, the two other most common systemic human fungal pathogens (Selmecki et al. 2006; Sionov et al. 2010; Ni et al. 2013). We feel we can exclude aneuploidy as an explanation for emerging resistance in *A. fumigatus* since, so far, no aneuploidy has been found in *A. fumigatus*. Aneuploidy in *A. nidulans* has been associated with mitotic instability and abnormal low-fitness phenotypes (Kafer and Upshall 1973) and also in *A. niger* stable aneuploids have never been observed (Debets et al. 1993). Furthermore, in some reports, aneuploidy in pathogenic fungi such as *Candida* was linked to the emergence of drug resistance, but was limited to fluconazole therapy. The prevalence of azole-

associated aneuploidy in these fungi appears not only to be due to increased azole therapy but also to the high plasticity of their genomes (Selmecki et al. 2010; Sionov et al. 2010; Kwon-Chung and Chang 2012). In contrast to the plastic *Candida* genome, the structure of the *Aspergillus* genomes appears rather stable (Gibbons and Rokas 2013). Further evidence that the observed resistance is due to changes in the DNA (mutations) rather than ploidy change comes from the observation of step-wise increases in resistance in the evolutionary trajectories. This is further supported by the results from a sexual cross of the ancestor and a resistant evolved strain, showing clear segregation of the resistant phenotype among the progeny (Supplement 3).

More generally, other parts of the *A. fumigatus* life-cycle could potentially provide alternative or additional ways to generate adaptive variation through the occurrence of recombination during the sexual and parasexual cycle (O'Gorman et al. 2008; Ene and Bennett 2014). Thus, whereas the abundant asexual reproduction may be significant for mutation supply, the much less common sexual and parasexual life cycle in nature could induce diversity through recombination (O'Gorman et al. 2008; Heitman et al. 2014). Furthermore, the parasexual life cycle also could promote adaptation by changing ploidy, as it appears to do in *A. nidulans* (Schoustra et al. 2007; Anderson et al. 2015). These possibilities provide interesting avenues for future research.

A scenario for the evolution of resistance in *A. fumigatus*

Based on the above reasoning, azole resistance in *A. fumigatus* could have evolved through the following scenario (Figure 5). All cultures were started with a conidiospore sampled from an ancestral azole sensitive colony that either may already contain rare random beneficial and detrimental mutations, or that developed mutations during asexual sporulation. This idea is supported by the fact that we found the same resistance levels (MIC value) after the first week of fungicide exposure of all cultures. After this first week, however, continued mycelial cultures did not further increase in resistance since potential beneficial mutations that appeared for instance during asexual sporulation on top of the mycelium were not able to spread and thus not available for selection.

In contrast, in the treatment including sporulation, all initial and subsequent mutations that occurred during sporulation could fix in those cultures and contribute to further resistance development, as seen in the stepwise increases of resistance. Continued mycelial growth ignored all these potential second and higher step mutations, because these mutations were less likely to happen in the first place, are at least partially recessive, and were trapped in a predominantly non-resistant mycelium, resulting in lower final levels of resistance at the phenotype level.

Asexual sporulation is a common reproductive mode for a diverse group of fungi that includes many medically, industrially, and agriculturally important species (Springer 1993; Adams et al. 1998; Mah and Yu 2006; Metz et al. 2011). Asexual sporulation is a conidiation process and a primary means of dispersion (Mah and Yu 2006; Metz et al. 2011) and can also occur within the lungs of patients. In our study, we illustrate asexual

sporulation to involve, (i) the alternation between multinucleate mycelium and uninucleate conidiospores which facilitates the expression of individual partly recessive mutations, and, (ii) the generation of large numbers of asexual conidiospores that increases the effective population size and mutation supply of a colony and that thus contributes to the diversity of populations. These combined effects of asexual sporulation may explain the rapid evolution of azole resistance in *A. fumigatus* as well as the overall diversity of *A. fumigatus* in the field and in hospitals (Samson 1994; Debeaupuis et al. 1997; Chazalet et al. 1998).

Relevance for azole-resistance development

Our results indicate that the risk of azole-resistance selection in *A. fumigatus* depends on the selection pressure - i.e. which specific azole was used - in an environment that allows asexual sporulation. A recent assessment by Gisi, indicated that high concentrations of azole fungicides are used for specific applications, including bulb dipping and protection of materials, such as wood (Gisi 2013). The risk of azole-resistance selection related to these applications, however, depends on the presence and exposure of sporulating *A. fumigatus,* which remains to be investigated. The second major application that, according to these principles, carries a high risk of resistance selection is azole therapy in animals and humans (Gisi 2013) as also azole therapy involves high exposure levels at the site of infection. Asexual sporulation of *A. fumigatus* was found in animal and human lungs with cavitary *Aspergillus* diseases such as chronic cavitary aspergillosis and aspergilloma, where vegetative hyphae are exposed to air (Figure 1C) (Adams et al. 1998). Clinical case series indeed show that resistance emerges exclusively when both conditions are present: during azole therapy of patients with cavitary lesions (Camps et al. 2012). In contrast, azole resistance selection during azole therapy has not been reported in patients with invasive aspergillosis (Dannaoui et al. 2004). Despite high azole exposure, the risk of azole resistance development in these patients appears much lower. Our results now support the interpretation that this is due to the absence of asexual sporulation in the patients.

Nevertheless, azole resistance has been reported in patients with invasive aspergillosis. Patients are believed to inhale already azole-resistant spores that then colonize the patient, rather than that the fungus with which they are infected develops resistance during azole therapy (Verweij et al. 2013). It indicates that these azole-resistant unicellular spores are more adapted to the patients' "ecosystem" than multicellular mycelium, which strongly supports the view that a regular unicellular life stage could promote the removal of deleterious mutations and the selection of beneficial mutations. Overall, it is essential that future research focuses on testing the role of asexual reproduction of the fungus in patients exposed to medical triazoles, as this may lead to new management strategies that avoid or overcome resistance selection.

Different azole fungicides have different activity against the ancestor (Supplement 4 MGR(b)=3.9±0.05mm/day; MGR(d)=3.7±0.05mm/day; MGR(e)=4.2±0.08mm/day; MGR(p)=5.6±0.11mm/day; MGR(t)=4.2±0.11mm/day;) and MIC (b)= 4; (d)=2; (e)=8;

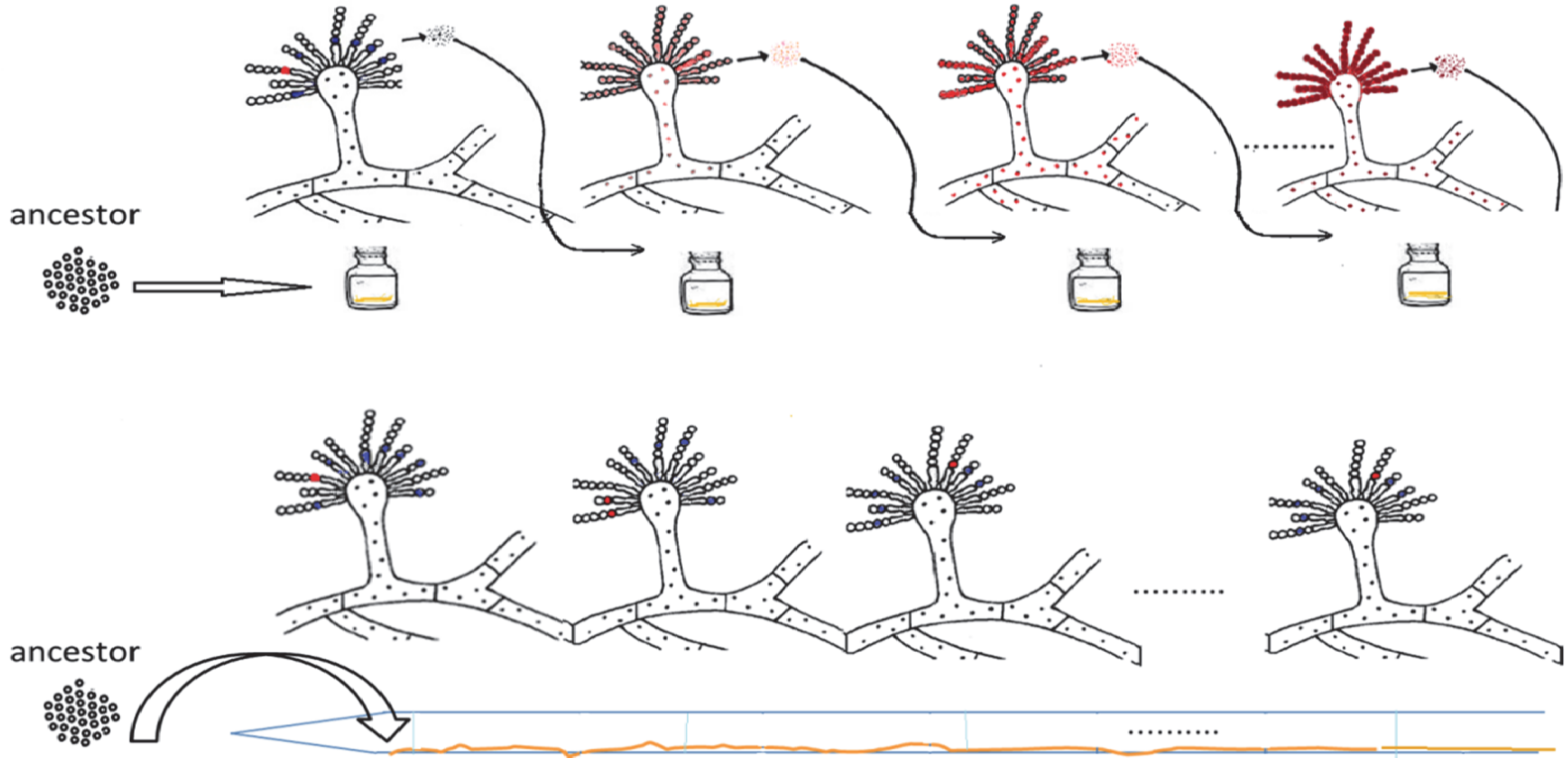

Figure 5. An evolutionary model for the development of azole resistance in *A. fumigatus* cultures

Upper panel: The cultures allowing for serial asexual sporulation (transfer of asexual spores produced by asexual sporulation)

Lower panel: The cultures without asexual sporulation (without a role for asexual spores).

Yellow line: medium with azole fungicides; red dots: nuclei carrying beneficial mutations; blue dots: nuclei carrying detrimental mutations. From pink to dark red: resistance level increase. Note that asexual spores are produced in both modes of culturing, but in the lower panel ("without asexual sporulation") the asexual spores produced do not germinate nor contribute to the development of azole resistance. Moreover, as the hypha contain multiple nuclei, resistance via mycelial growth alone is unlikely to emerge over relevant time scales, such as during patient treatment.

(p)=8; (t)=4). The initial fitness of the ancestor to five azole fungicides was inversely proportional to the rate of adaptation of the ancestor to five azole fungicides (supplement 5), which may explain the differences in the selection level of the different fungicide azoles against *A. fumigatus*. Azole resistance in our lineages is incomplete dominant, and explaining this observation requires knowledge of the mechanism(s) involved. From the evolutionary trajectory and sexual cross analyses it appears that the resistant strain contained two mutations (Supplement 3). Maybe epistatic interaction between these mutations may explain the incomplete dominance. In relation to this, it would be of interest to test the predominant highly resistant mutant TR_{34}/L98H found in hospitals for dominance.

The clear correlation between MIC and MGR is also useful for future studies on the development of resistance in fungi. For susceptibility testing, the MIC value of azoles against *Aspergillus* are influenced by variable factors such as inoculum size, endpoint and reading time, which can easily result in a 1–2 wells difference in the MIC interpretation (Gehrt et al. 1995; Llop et al. 2000; Espinel-Ingroff et al. 2001; Albarrag et al. 2011). Also it is difficult to detect relatively small differences in the level of resistance. In contrast, MGR is much more accurate and easier to measure and can thus be used as a reliable proxy to determine the resistance level. However, for the cultures of the treatment without asexual sporulation, we did not find a correlation between MIC and MGR, which is likely explained by the lower levels of MIC and MGR increase during the evolution experiment. Probably there are mutations that affect MIC but not MGR at one particular level of drug.

Conclusions and Future Outlook

Our results demonstrate that the full life cycle of *A. fumigatus* needs to be taken into account to explain the emergence and possible persistence of azole resistance. In addition, the selection pressure appears to be a key factor. As we observed differences in selection pressure for the various azole compounds, the implications of both the dose and the molecule structure should be considered in future studies. While knowing the exact identity of the adaptive mutations in *A. fumigatus* was not required to address the hypotheses in this paper, it will be an essential avenue of future research, which will further unravel the evolutionary dynamics of *A. fumigatus* adaptation both in the human setting and in the environment. Understanding the key factors that facilitate resistance selection in *A. fumigatus* is essential to design strategies that prevent or overcome this emerging threat.

Acknowledgements

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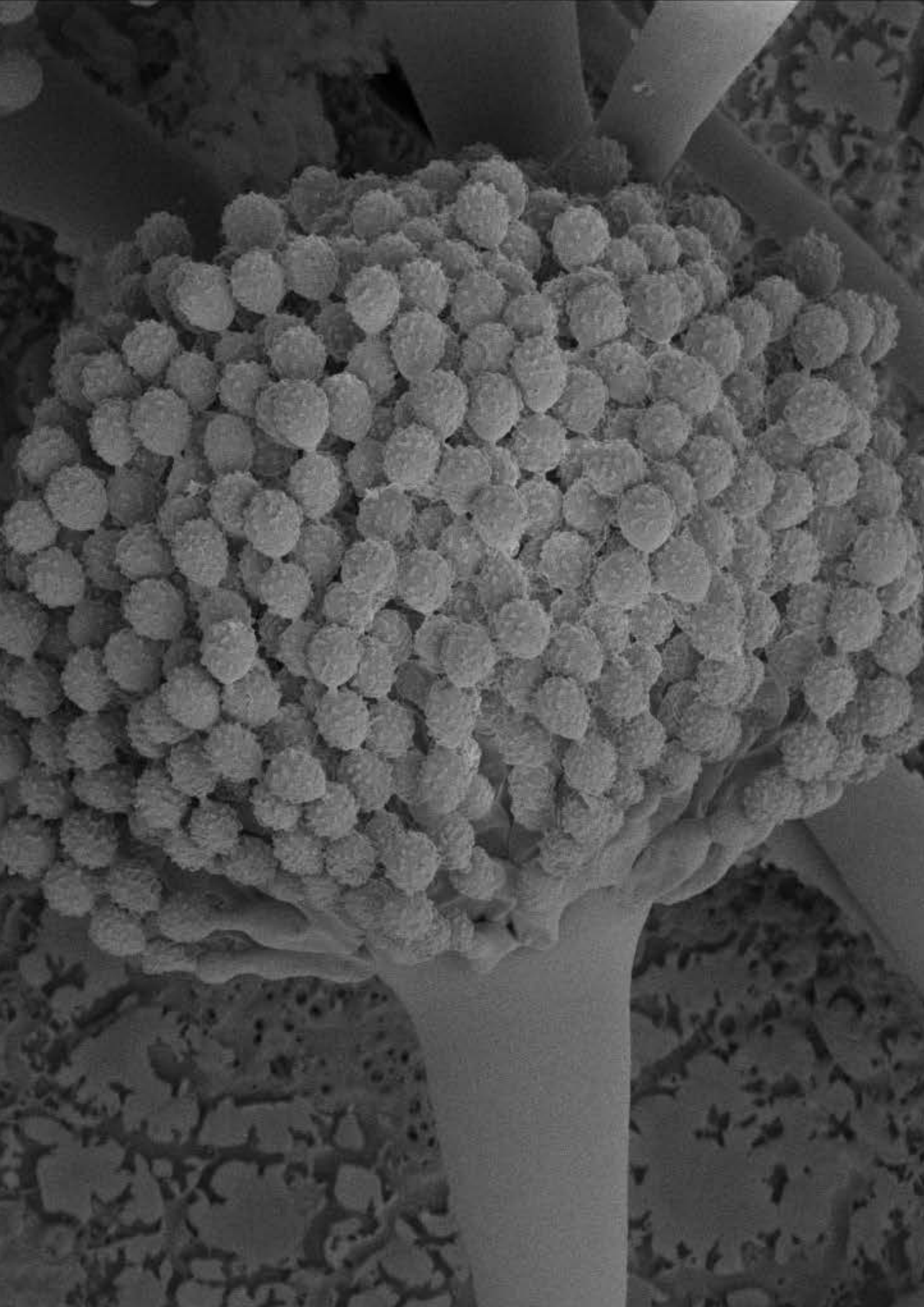

CHAPTER IV

Evolution of cross-resistance to medical azoles through exposure to agricultural azoles in *Aspergillus fumigatus*

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Abstract

Resistance to azoles in *A. fumigatus* is an emerging problem for immunocompromised patients. There are currently two presumed routes of the emergence of resistance to medical azoles: (1) through selection pressure of azoles from medical drugs in human patients and (2) through the selection pressure for resistance from the use of azole fungicides in agricultural environments. To assess the potential of selection of resistance to medical drugs in agricultural environments, we performed an evolution experiment with *A. fumigatus* under selection pressure of one of five different agricultural azoles and assessed cross-resistance to three medical azoles. We found wide-spread cross-resistance indicating that resistance to medical azoles can arise through the selection pressure of agricultural azoles. All evolved lineages showed nearly exactly the same evolutionary dynamic to agricultural azoles and medical azoles, strongly suggesting that the same mutations induce resistance to both agricultural and medical azoles. From the five agricultural azoles we used, difenoconazole exerts highest selection for overall azole resistance, especially for inducing high resistance to itraconazole. We observed dynamic phenotype changes (morphotype and fitness) over the course of the evolution experiment, which included the colony size (big and small), colony textures (dense and fluffy) and sporulation time (early or late). Whole-genome sequencing revealed that mutations in several targets are involved in the resistance mechanism.

Key words: *Aspergillus fumigatus*; experimental evolution; agricultural and medical azoles; azole resistance; evolutionary trajectory.

Introduction

Azole resistance is an emerging health problem in the saprophytic mold *Aspergillus fumigatus.* This fungus is responsible for the vast majority of invasive fungal infections in immunocompromised patients, and has spread globally in the last decades (Bignell 2014; Cuenca-Estrella 2014). (Latgé 1999; Rhodes 2006; Lockhart et al. 2011; van der Linden et al. 2011; Chowdhary et al. 2012a; Snelders et al. 2012; Vermeulen et al. 2012; Astvad et al. 2014; Bignell 2014; Rocchi et al. 2014b; Lavergne et al. 2015; Wiederhold et al. 2015; Patrice Le et al. 2016). Triazoles are the class of antifungal drugs used to treat human infections; itraconazole, posaconazole and voriconazole are the most widely used (Albarrag et al. 2011). These triazoles inhibit the enzyme sterol 14α-demethylase, which is encoded by the *cyp51A* gene, thereby blocking its function in the ergosterol biosynthesis pathway. This results in ergosterol depletion and accumulation of toxic sterols (Joseph-Horne and Hollomon 1997). The most common resistance mechanism known includes the alteration of the *cyp51A* gene (Howard et al. 2006; Howard et al. 2009; Bueid et al. 2010; van der Linden et al. 2011 (Mellado et al. 2007; Snelders et al. 2008; Mavridou et al. 2010; Snelders et al. 2010; Chowdhary et al. 2012b; Pelaez et al. 2012; Rath et al. 2012; Rocchi et al. 2014a; Wiederhold et al. 2015)). In the last decade, widespread resistance emerged against itraconazole, and more recently to voriconazole, and numerous resistant strains have been isolated from patients.

Interestingly, highly resistant *A. fumigatus* is also found from soil, flowerbeds and other agricultural environments (Snelders et al. 2009). In fact, azole fungicides are widely used in agricultural settings to control various plant pathogens. Agricultural azoles are triazole derivatives and are unique in that they are the only class of antifungals that are used both in agriculture and in clinical medicine (Verweij et al. 2009b; Snelders et al. 2012; Stensvold et al. 2012). The volume of agricultural triazoles sold has almost doubled between 1995 and 2007 to 130000 kg per year in the Netherlands alone, against a yearly use of around 400 kg of medical azoles (Verweij et al. 2009b). Resistant *A. fumigatus* isolates from patients and the environment share the repeated occurrence of mutations in the *cyp51A* gene cluster as the resistance mechanism (Domsch et al. 1980; Gisi 2013). This suggests that there are potentially two routes of selection for resistance exist in *A. fumigatus* strains found in patients (Verweij et al. 2009b; Verweij et al. 2013). The first route is through patients with chronic aspergillus disease as CF patients, who are under prolonged treatment with medical azoles. This medical route to resistance has been demonstrated experimentally (Camps et al. 2012b). The second route is through exposure of *A. fumigatus* to agricultural azoles used in crop protection, resistance to which could give rise to cross-resistance to medical azoles. Several studies have suggested that azoles used in agricultural settings can select for cross-resistance to medical azoles based on the shared mechanism for resistance (Snelders et al. 2012; Stensvold et al. 2012; Chowdhary et al. 2013; Verweij et al. 2013; Bowyer and Denning 2014; Faria-Ramos et al. 2014). For instance, Snelders et al. (2012) discussed the high

resistant strain (isolated from a medical environment) carrying the mutations TR_{34} /L98H showed resistance to both agricultural and medical azoles and that agricultural and medical azoles target the same active sites in enzymes of the ergosterol pathway of *A. fumigatus.*

However, there is no direct evidence that exposure of *A. fumigatus* to agricultural azoles can lead to cross-resistance to medical azoles. In a previous study that focused on the effects of different modes of fungal reproduction on resistance development, we had evolved *A. fumigatus* strains over seven weeks in the presence of one of five agricultural fungicides (Zhang et al 2015). In the present study, we assay these evolved strains for cross-resistance to three medical azoles. We further ask whether the increase in resistance to medical azoles mirrors the observed evolutionary dynamics of resistance development to agricultural azoles. Finally, for several evolved populations, we link fitness to population dynamics and to observed mutations at the genome level. This information is essential to understand the potential routes to medical azole resistance development and associated genetic changes under the selection pressure of agricultural azoles.

Materials and methods

Fungal isolates and azoles

All evolved fungal cultures used in this study are from a previous study that focused on the role of various modes of fungal reproduction in adaptation (the development of resistance to agricultural azoles) (Zhang., et al 2015). In that previous study, we evolved ancestor *A. fumigatus* CBS 140053 in the laboratory over seven weeks*.* Six replicate lineages evolved in the presence of one of each of five agricultural azoles and six lineages evolved without any agricultural azole as a reference (called control lineages). For one of the reproductive conditions, we weekly transferred a sample of fresh asexual spores to fresh medium. The stored samples for each lineage at each of the seven time points are used in the present study. Thus, the total number of samples was 253 ((5 azoles+control)*6 replicates* 7 time point + ancestors = 253).

The five agricultural azoles (AGRO) used for the selection were bromuconazole, tebuconazole, epoxiconazole, difenoconazole and propiconazole. In the present study we used the medical azoles itraconazole (ITR), voriconazole (VOR) and posaconazole (POS). All azoles were purchased from Sigma Company.

Susceptibility testing of evolved cultures to the medical azoles

We assayed the level of resistance of all lineages that had evolved in the presence (or absence) of one of five different agricultural azoles over seven weeks against the three

medical azoles. We performed resistance test following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) reference method (Lass-Flörl et al. 2008). Briefly, resistance level was defined as the minimal inhibitory concentration (MIC) that results in 100% growth inhibition of fungi. The relative MIC was defined as the individuals' MIC divided by the MIC of the azole sensitive ancestor *A. fumigatus* CBS 140053. RPMI-1640- 2% G medium (Sigma) was supplemented with each of the medical azoles in concentrations ranging from 0.016 to 16 mg/L in two-fold concentration steps and loaded into a 96-well plate. Spore suspensions used to initiate the assay were prepared from fungal colonies grown on solid medium using saline-Tween (0,8% HCl with 0.05% Tween 80 – Sigma- in water). We estimated the density of spore suspensions on a Coulter counter to standardize the concentration of spores to 1-2.5×10^5 CFU/mL prior to the MIC assay and then inoculated the standardized spore suspension into flat-bottomed 96-well microtiter plates containing a series with increasing amounts of and incubated at 37°C for 48 h. We performed readings from the bottom of the microtiter plates using a mirror and determined at which concentration we observed 100% inhibition (Camps et al. 2012b). There were six replicates for each assay. If at a concentration of 16 mg/L no inhibition was observed, we (arbitrarily) classified the MIC as 32 mg/L. We measured the MIC values for each time point for each lineage, allowing to generate an evolutionary trajectory of relative MIC. In these trajectories, stepwise increases in MIC are indicative of the fixation of a mutation conferring resistance (Lenski and Travisano 1994).

Population dynamics in the evolutionary lineages over time

We selected three lineages from the difenoconazole treatment that achieved the highest resistance increase (lineages D1, D3, D6) for a more in-depth study of the evolutionary dynamics looking at morphological changes. Around 100 spores from samples from different time points of each lineage (D1, D3, D6) were spread onto MEA plates with 1µg/ml of difenoconazole. The morphology of resulting fungal colonies was analyzed using the Z7 programme and proportion of each type was recorded after two days of growth. Various different morphologies were observed, each of which was inoculated into bottle with 1µg/ml difenoconazole, incubating at 37°C for a week to assay total spore production. To measure spore production, we harvested spores from each bottle by adding two mL of saline-Tween and beads into bottle and vortexed for five minutes. The spore production was assayed at Coulter counter (Schoustra et al. 2009) after filtering out large pieces of hyphae. Further, the mycelial growth rate (MGR) of each morphological type was measured on MEA plates supplemented with 1µg/ml difenoconazole.

Whole-genome sequencing

We performed whole-genome sequencing on each morphological type found in lineages D1, D3 and D6 (described above). Furthermore, we sequenced three lineages that had

evolved in the absence of azoles (C2, C4, C5). For sequencing, we picked three colonies of the same morphotype and performed a combined DNA extraction. The sequencing was performed by BGI.

Fastq files were analysed for quality using Fastqc (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). We further counted the number of reads and number of nucleotides of all samples, which were well balanced (see table S1). Since most nucleotides had a quality higher than 30 (Q>30) we trimmed the reads using 30 as minimum quality and 70 nucleotides as minimum length (trim-fastq.pl from the popoolation pipeline (Kofler et al. 2011a)). Reads were aligned using BWA mem (v. 0.7.10, -B 4, -O 6, -E 1(http://bio-bwa.sourceforge.net/bwa.shtml)) to a de-novo assembled genome, which was produced using only reads from the ancestor sample (Celera Assembler, 8.3rc1(Myers et al. 2000)). Aligned reads were filtered for quality (MAPQ > 50) and only reads with both ends aligned were kept. Then we used picard tools (1.107(http://picard.sourceforge.net.)) to remove duplicates and GATK (3.3-0(McKenna et al. 2010)) to realign around indel regions. Then we calculated pairwise fst values between all evolved lines and the ancestor (fst-sliding.pl, popoolation2(Kofler et al. 2011b)). Because we combined DNA of three clones, we essentially estimated the allele frequencies of 0, 1/3, 2/3 or 1. We called SNPs by selecting loci with a pairwise fst larger than 0.05, with a coverage higher than 25X. We then estimated whether a SNP was in a gene and if so, non-synonymous by blasting the variant and ancestral sequence around the SNP (blastx, ncbi database, only for *A. fumigates*). An additional analysis was done to detect structural variants using breakdancer (http://www.nature.com/nmeth/journal/v6/n9/abs/nmeth.1363.html, v 1.1.2) for which we used unfiltered BAM files. All variants found were visually inspected using IGV (James et al 2001, v. 2_3_57). SNPs that were omitted were often found to lay in regions rich in SNPs and in which multiple SNPs were found within single reads.

Statistical analysis

To explain the observed variation in relative MIC of the evolved strains and control against different azoles (one agricultural and three medical azoles), we performed the Analysis of Variance (ANOVA) for the relative MIC data with type of agricultural Azole (Azole) and Resistance to three medical azoles (Test) as fixed factors (model: Relative MIC ~ Azole*Test). Furthermore, because the effect of relative MIC depended on the azole used for the selection (see results), we performed post-hoc LSD tests to check whether the achieved level of resistance depended on the azole fungicide used for selection (model for each test azole separately: Relative MIC~ azole treatment) and which medical azole were used to test (relative MIC~ medical azoles test).

The analysis of the relative MIC are presented as mean values ± standard error of the mean (SEM). p-values of ≤ 0.05 (*) were defined as significant. To test for a correlated response between an increase in resistance to the agricultural azole used for selection and cross-resistance to the medical azoles, we constructed fitness trajectories in all four environments. From these trajectories, we recorded the timing of an increase in

resistance (MIC) and we used linear regression asking whether there is a correlation in the timing of an increase in resistance to the agricultural azole and cross-resistance to the medical azole.

Results

Susceptibility testing of evolved cultures against agricultural and medical azoles

We assayed the cross-resistance level to three medical azoles (itraconazole, voriconazole, posaconazole) of all lineages that had evolved under laboratory conditions in the presence (or absence) of one of five agricultural azoles at each of the seven evolutionary time points. The level of resistance of evolved cultures against agricultural azoles had been assayed in a previous study (Zhang et al 2015). Figure 1 shows the evolutionary dynamics of resistance level (defined as the Minimal Inhibitory Concentration of a given evolved culture relative to the MIC of the ancestor) averaged for the six replicate lineages that had evolved under the same conditions. Figure 1A shows data of resistance to the agricultural azole used for selection (taken from Zhang et al 2015), figure 1B-D shows the cross-resistance to medical azoles. Statistical analyses show that on average evolved lineages have an increased resistance to agricultural azoles (Azole), as well as an increased cross-resistance to the three medical azoles (Test) and that this was dependent on the medical azole used for test (Supplementary table 1A). The resistance (relative MIC values) against the three medical azoles increased by 2-3.3 fold (POS), 3-4.6 fold (VOR), strikingly 4.4-35 fold(ITR) at the end of seven weeks. This cross-resistance is in the same other of magnitude or higher than the resistance increase to the agricultural azole that was used during the seven week selection. The relative MIC of itraconazole against evolved cultures were significantly higher than the MIC of posaconazole and voriconazole (Supplementary table 1B)

The five agricultural azoles that we used during the selection for resistance induced different levels of cross-resistance to the three medical azoles. In particular, for the lineages evolved under the selection pressure of difenoconazole, the cross-resistance against the three medical azoles is significantly higher than the other four agricultural azoles (Supplementary table 1C). These differences suggest that difenoconazole is a stronger selective agent for the evolution of resistance to medical azoles.

According to the medical-resistance breakpoints of different azoles for *A. fumigatus* using the proposed EUCAST susceptibility testing methodology, a strain with either MIC(posaconazole) > 0.5 mg/L, MIC(voriconazole) > 2mg/L or MIC(itraconazole) > 2mg/L is considered resistant to that medical drug (solid lines in Figure 1B-D)(Verweij et al. 2009a). While all lineages have increased their resistance to medical azoles over seven weeks, not all are now resistant in a clinical sense, i.e. have a resistance above the resistance boundary. Counting individual lineages, we obtained out of 253 cultures a

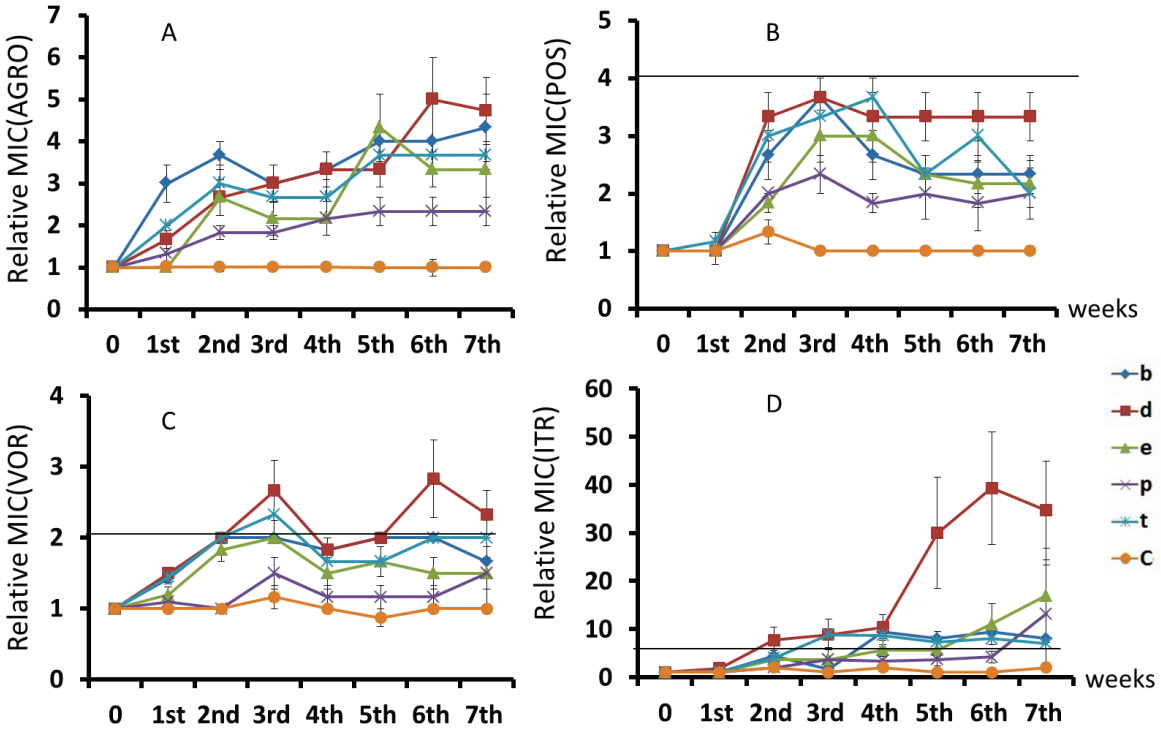


Figure 1. Evolution of resistance over seven weekly transfers

Each line shows the average of six replicate lineages that had evolved in the presence (or absence) of one of five agricultural azoles. Error bars indicate the standard error of the mean (SEM). Resistance is given by the relative MIC-value of an evolved strain relative to the ancestor. Panel A: Resistance of evolved lineages against the agricultural azole used for the selection; each series of six lineages assayed for resistance against their respective azole used for selection (data from J Zhang et al 2015) b = bromuconazole, t = tebuconazole, e = epoxiconazole, d = difenoconazole, p=propiconazole, c= no azole present. Panel B: Cross-resistance of evolved lineages against the medical azole posaconazole (POS). Panel C: Cross-resistance against the medical azole voriconazole (VOR). Panel D: Cross-resistance against the medical azole itraconazole (ITR). In panels B to D the horizontal solid line shows the boundary above which strains are considered resistant by clinical standards (resistance breakpoint).

total of 97 that are resistant to itraconazole, eight to voriconazole and no resistant cultures to posaconazole (Table 1). This is in line with the results described above that indicate difenoconazole imposes the strongest induction of cross-resistance to medical azoles (Figure 1). These results further suggest that posaconazole is the most effective medical azole with the least cross-resistance from agricultural backgrounds and itraconazole is the least effective. Notably, this matches with time since these medical triazoles were clinically licensed: itraconazole in 1997, voriconazole in 2002 and posaconazole in 2006 (Denning et al. 2014; Siopi et al. 2014).

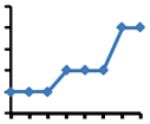
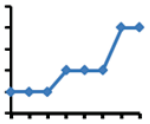
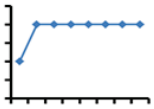
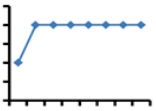
Table 1. The number of individual lineages that evolved cross-resistance to different medical azoles at a level of clinical resistance (resistance breakpoint, Figure 1) after selection on an agricultural azole

	Number of resistant cultures		
Stress (Agricultural fungicides)	MIC(POS) > 0.5mg/L	MIC(VOR) > 2mg/L	MIC(ITR) > 2mg/L
Bromuconazole	0/42	0/42	26/42
Difenoconazole	0/42	6/42	25/42
Epoxiconazole	0/42	0/42	17/42
Propiconazole	0/42	1/42	8/42
Tebuconazole	0/42	1/42	21/42
Total	0	8	97

Evolutionary patterns of evolved cultures on agricultural azoles and correlated response on medical azoles

Under the selection pressure of five agricultural azoles, the resistance level of each lineage to different azoles increased over the seven-week selection period through the successive fixation of mutations. This results in a step-wise increase in resistance (relative MIC) and fitness over time, as is typically observed in laboratory experimental evolution (Lenski and Travisano 1994; Schoustra et al. 2009). We measured resistance (Relative MIC) against the agricultural azole used for the selection and cross-resistance against the three medical azoles and recorded the time points at which an upward step in resistance occurred (Table 2). We compared the evolutionary trajectories over time for the timing and the number of step-wise increases of resistance, asking whether a fixation of a mutation to the agricultural azole leads to a parallel increase in cross-resistance to the three medical azoles. Roughly half the lineages (D1, 3, 6, E4, P3) went through two steps to reach high fitness and half (D4, B5, B6, T4, E1) went through one step. Linear regression analysis shows that lineages on average exhibit the same step wise increase in resistance to the agricultural azole used for the selection and cross-resistance to the three medical azoles (correlation coefficients with statistical significance between of the timing of resistance increase of agricultural azoles and the medical azoles, see Supplementary table 2A, B). This parallel increase in resistance suggests that the genetic changes that underlie the increased resistance to agricultural azoles also lead to cross-resistance to medical azoles.

Table 2. Evolutionary trajectories of evolving lineages against agricultural and medical azoles

Evolutionary trajectory pattern	Pattern of agricultural azole	Pattern (POS or VOR or ITR)
Two steps 50%		
One step 50%		

Population dynamics within three evolutionary lineages

Lineages D1, D3, D6 (three lineages that evolved in the presence to difenoconazole) reached high (cross-) resistance after seven weeks of experimental evolution and were selected for whole-genome sequencing. Prior to sequencing, we performed an in-depth analysis of population dynamics for these three lineages to find out whether or not the lineages diversified into different types by diluting and plating the lineages cultures from different time points. We found that the lineages consisted of a mixed population of different morphological types.

In lineage D1, three morphological types appeared: ancestor, middle size and big size (Figure 3). The ancestral morphotype declined from the first week and was completely replaced by the middle size morphotype at the fifth week that had occurred at the second week. It declined sharply at the sixth week and disappeared at the seventh week, when it was replaced by the big size colony that had occurred at the fifth week. The middle size colony appears to be the intermediate type. The shift in morphotypes suggests the middle size colony carries at least one beneficial mutation compared with the ancestral type and that the big type carries at least two mutations compared with the ancestor. This idea is supported by the outcome of a sexual cross between D1-big and a derived ancestor showing segregation of at least two loci among the progeny (Supplementary Figure 5). This is in line with an increased resistance to both difenoconazole and the medical azoles (MIC resistance test) and increased growth rate (MGR) compared to the ancestral type (ANOVA: $F_{2,6}= 1463.769$, $P < 0.01$, Post-Hoc LSD test, $P_{ancestor\text{-}middle\text{-}big} < 0.01$ Supplementary 3A), and increased spore production (ANOVA: $F_{2,6}= 69.095$, $P < 0.01$, Post-Hoc LSD test, $P_{ancestor\text{-}big} < 0.01$, $P_{ancestor\text{-}middle} = 0.891$) (Figure 3, Supplementary table 3B).

In lineage D3, two morphological types (dense and fluffy) occurred during the evolutionary experiment (Figure 3). The ancestral type declined sharply after the first week and was completely replaced by the dense morphotype at the third week. Between the third and fifth week dense morphotype declined in frequency to reach 35% at the seventh week. In the third week, a fluffy morphotype occurred and reached a frequency of 70% at the seventh week. Resistance level to difenoconazole and cross-resistance to

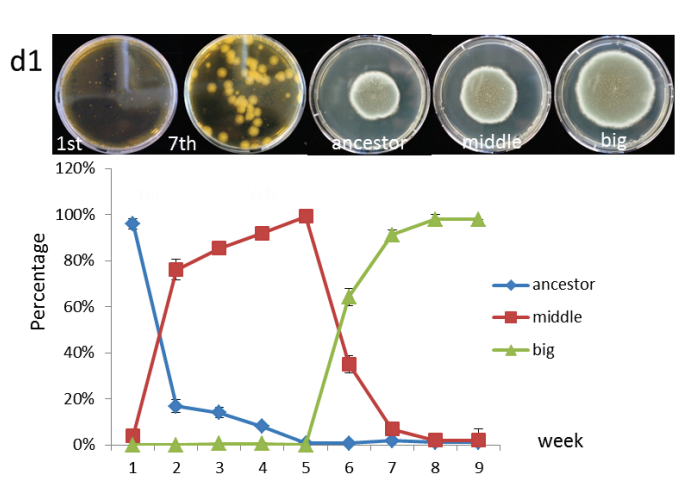
the three medical azoles show that the mixed population of fluffy and dense morphotype is more resistant to itraconazole and difenoconazole (MIC resistance test) than the morphotypes in isolation. The growth rates (MGR) of three types of colonies showed the fluffy colony had faster growth rate than other two types (ANOVA: $F_{2,6}$= 10299, P<0.01, Post-Hoc LSD test, $P_{ancestor\ -dense\text{-}fluffy}$ <0.01, Supplementary table 3A). The dense morphotype colony could produce significantly more spores than other two types (ANOVA: $F_{2,6}$= 586.204, P <0.01, Post-Hoc LSD test, $P_{ancestor\text{-}dense}$ <0.01; $P_{ancestor\text{-}fluffy}$ <0.05) (figure 3, Supplementary 3B).

Within lineage D6, we observed four morphotypes. The ancestral type sharply declined to zero, to be replaced by a fluffy morphotype by the third week. This fluffy type occurred since the second week and completely fixed at the third week, followed by a decline in the fourth week and then remained stable at a frequency of 60%. A big size morphotype appeared at the second week, reached a frequency of 20% before disappearing in the fifth week. The white morphotype has a sporulation defect and occurred at third week and reached a stable frequency of 40%. Resistance level to the three medical azoles was highest for the mixture of fluffy and white type is better fit in high concentration of itraconazole and difenoconazole (MIC resistance test). The growth rate (MGR) of four types of colonies showed the fluffy type had faster growing than other three types (ANOVA: $F_{3,8}$= 1771.202, P<0.01, Post-Hoc LSD test, $P_{ancestor\text{-}big\text{-}fluffy}$ <0.01; $P_{ancestor\text{-}white}$ =0.103, Supplementary table 3A). During culturing, this white morphotype does not sporulate until the fifth day of culturing and in the end produces significantly 10 times less spores than the ancestral morphotype (ANOVA: $F_{3,8}$= 771.498, P<0.01, Post-Hoc LSD test, $P_{ancestor\text{-}fluffy\text{-}white}$ <0.01; $P_{ancestor\text{-}fluffy}$ <0.05, Supplementary table 3B).

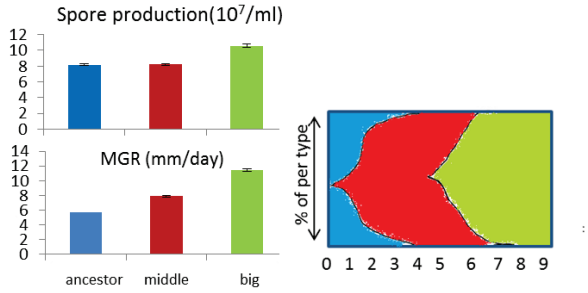
Whole-genome sequence analysis

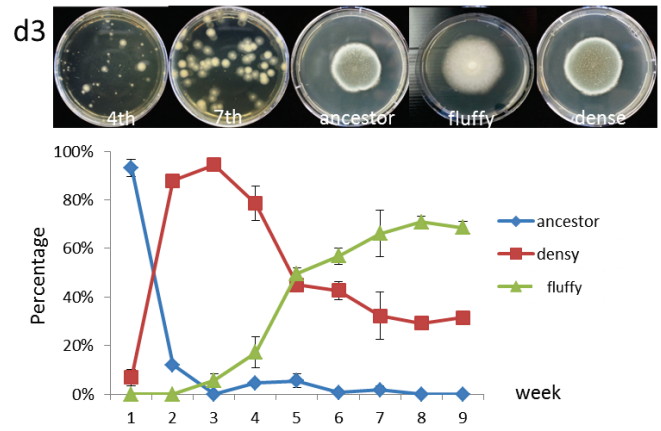
We sequenced a total of eleven (9) genomes: the ancestor, three lineages that had evolved in the absence of azole for seven weeks (C2, C4 and C5 called control lineages) and the five morphotypes observed in the lineages D1, D3 and D6 (described above). We used the sequence of the ancestor of the evolution experiment for a *de novo* genome assembly.

We found mutations in all evolved lineages. All SNPs found were visually inspected in IGV (version IGV-2.3.57) using the *de novo* assembled genome, all samples and filtered bam files. SNPs that were kept after visual inspection are listed in Table 3. All SNPs found were non-synonymous and invariable in the ancestor, indicating that all SNPs were new for the evolved lines. Next to SNPs one deletion (TG) was found in the control 5 lineage, which caused a frameshift with stop codon in a conserved hypothetical protein. In the three difenoconazole lineages, we found different morphotypes evolved during the evolutionary experiment. After sequencing analysis, lineages that evolved under the

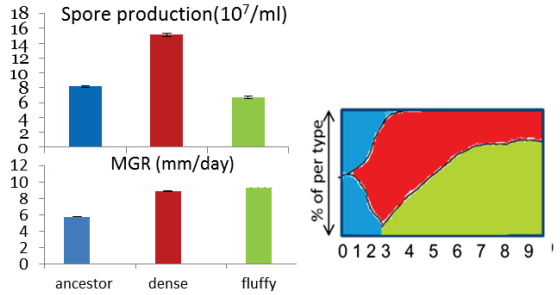


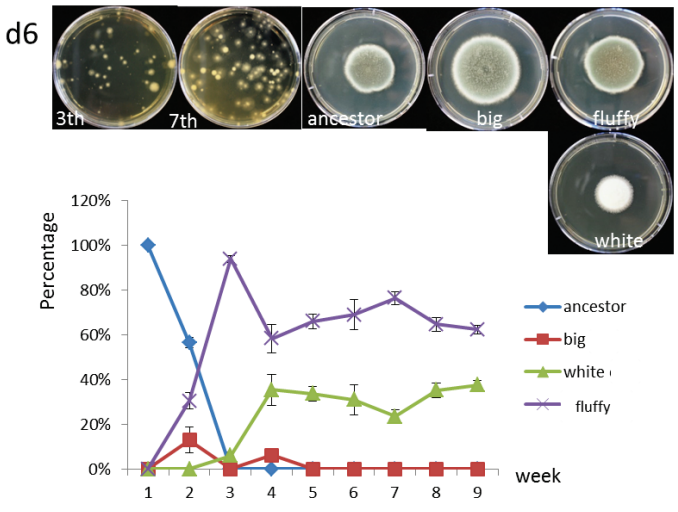
MIC	MIC(D)	MIC(POS)	MIC(VOR)	MIC(ITR)
Ancestor	4	0.25	0.5	0.5
Middle	16	0.5	1	8
Big	32	0.5	4	32





MIC	MIC(D)	MIC(POS)	MIC(VOR)	MIC(ITR)
Ancestor	4	0.25	0.5	0.5
Dense	8	0.5	1	8
Fluffy	8	0.5	2	8
Mixed	16	0.5	2	16





MIC	MIC(D)	MIC(POS)	MIC(VOR)	MIC(ITR)
Ancestor	4	0.25	0.5	0.5
Fluffy	8	0.5	2	8
Big	16	0.5	1	8
White	8	0.25	2	8
Mixed(F&W	16	0.5	2	32

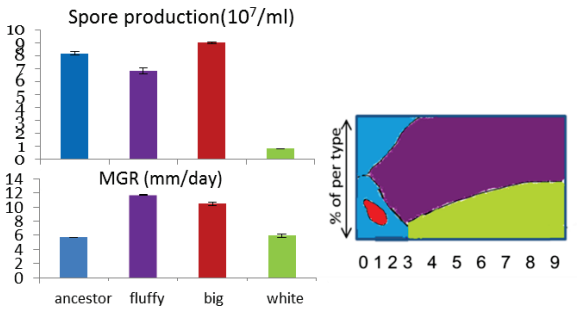


Figure 3. Population dynamics and characteristics (frequency changes, growth rates, spore production and MIC value) of different types in in three difenoconazole lineages.

pressure of azole treatment and lineages that evolved without azoles both acquired mutations indicating adaptations of the strain to the laboratory environment, which might not be related to resistance increase (Supplement 4). Except these mutations, D1-big size colony contained one CYP51A mutation, a gene known for its potential to harbor resistance mutations. None carried a mutation in the HapE gene, which is another target gene for resistance (Camps et al. 2012a). Others are with potential new resistance mechanisms (Table 3). D1-big with the cyp51 mutation had higher fitness (in terms of MGR and spore production). Further, D1-big carries another mutation in HMG CoA reductase, as is expected since there was an intermediate type (D1-middle) between D1-big and the ancestor. The P ->L substitution at amino acid 320 in HMG CoA reductase was found to be completely divergent in D1-big and D3-dense compared with the ancestor, but as well in the control 5 line, suggesting that this mutation may be a general adaptation to laboratory conditions with an associated increase in azole resistance. In the control 2 line we found three SNPs that were still variable, of which one was also found in d3 fluffy (374 P-> S in Putative amidohydrolase ytc J). These two SNPs are found in both evolved and control lines and are likely due to general adaptations to laboratory conditions. However, beside the P320L change in the HMG CoA reductase in D3-dense, we did not find any additional SNP. While this SNP was also found in the control 5 line, this line also contained a deletion which caused a frame shift and STOP codon in a conserved gene. This means that the control 5 and d3 dense are genetically different. Furthermore, the alternation in the HMG-CoA reductase, a key enzyme in mevalonate biosynthesis, rise the ergosterol has been reported in the *A. fumigatus* and *Candida* species (Macreadie et al. 2006). In D3-fluffy we found two additional SNPs that were not fixed (yet) that were not found in any other sample. Similarly, in D6-fluffy three new SNPs were found (compared to ancestor) that were private for D6-fluffy, while in d6white we found two private SNPs that were completely fixed. Trace-back experiment confirmed the PtaB Q264STOP is related to phenotype of white (later sporulation), while Hypothetical protein G167D is associated with the increased azole resistance. This co- balanced mixed population within D3 and D6 lineages can be explained by cross- feed or complementation between these two phenotypes. The function of each type in this co-balanced population needs further study. Also, to firmly establish that the mutations we detected through sequencing are the mutations responsible for increased resistance, a formal genetic basis would have to be established to knock-outs and complementation tests. This is beyond the scope of this current study.

Discussion

In this study, we tested the consequences of prolonged exposure of *A. fumigatus* to one of five agricultural azoles for cross-resistance to three medical azoles. We found widespread cross resistance to medical azoles in experimental populations after seven weeks of laboratory evolution under the pressure of agricultural azoles. We observed

that evolutionary lineages show similar evolutionary patterns to agricultural and three medical azoles, which provides strong evidence that use of agricultural azoles is responsible for cross-resistance to medical azoles. Difenoconazole imposed high selection for cross-resistance, in particular to itraconazole. We further detected population dynamic changes in evolving lineages, with lineages diversifying into several morphological types. Whole-genome sequencing revealed the presence of a mutation known for its effect of increased azole resistance (G138S mutation in the Cyp51 gene) as well as several other point mutations.

Table 3: Whole-genome sequence analysis of evolved strains

Evolved strain	Substitution (SNP/del)	Gene
D1 big	G138S (SNP) P320L (SNP)	CYP51 HMG CoA reductase
D3 fluffy	S109L (SNP) A276T (SNP)	TreA / Ath1 DUF1479 domain protein
D3 dense	P320L (SNP)	HMG CoA reductase
D6 fluffy	Q107STOP (SNP) G129V (SNP) VK 751--(DEL)	TOM70 MnSOD Mdm31
D6white	Q264STOP (SNP) G167D (SNP)	PtaB Hypothetical protein
Control 5	P320L (SNP) **HRTVIISP551-558 QNCYHFASTOP (DEL)**	HMG CoA reductase Conserved hypothetical protein

Note: Con5 contains an additional deletion (TG) that causes a frame shift (adding stop codon) in a conserved hypothetical gene (see table in supplement).

Interestingly, the resistant strains showed the same step-wise pattern of increase in resistance to agricultural azoles and medical azoles. Step-wise increases in fitness change are indicative for the fixation of a mutation (Lenski and Travisano 1994). In other words, this strongly suggests that mutations conferring resistance to agricultural azoles also confer cross-resistance to medical azoles. This was expected since the medical and agricultural azoles share the same site of action (binding site) and is structurally similar (Snelders et al. 2012). These results provide direct evidence that agricultural azoles can induce cross-resistance to medical azoles in *A. fumigatus* and is consistent with previous findings that mutations in the cyp51 gene (G138S) confer resistance both to agricultural as well as medical azoles (Chassot C 2008; Gisi U 2008; Snelders et al. 2012).

There has been a long standing debate about the interaction between environmental and clinical routes to azole resistance (Hof 2008; Gisi 2013; Lago et al. 2014). *A. fumigatus* grows and sporulates frequently in natural habitats such as soil and decaying plant material, places that might be contaminated with all kinds of azoles used to control a wide variety of fungal plant pathogens. Our work shows that resistance development to medical azoles through exposure in the environment is a plausible route. It has been suggested that the development of resistance in agricultural environments is less likely

than in medical environments due to the 10 to 100 fold lower concentrations of azoles used in agriculture in comparison to medical environments (Gisi and Sierotzki 2008; Kuck 2012). This exposure to low concentrations does not limit the emergence of resistance: our results show that even with azole concentrations as low as 1 µg/ml up strains resistant to 40 fold higher concentrations can emerge. Therefore, a high concentration is not an essential factor for resistance development. In fact, the higher number of growth cycles and associated opportunity for mutations to arise could actually enhance resistance development (Zhang et al 2015) and agricultural fields with relatively low maximum exposure are also highly likely to induce such high stress levels to promote the development of azole resistance. Decaying plant material of different origins, such as compost, self-heated hay and corn heaps, and litter of, for example, ferns, cotton, barley, cabbage and conifers are important habitats for *A. fumigatus*. These materials are likely to contain sufficient (residual) agricultural azoles to generate a selection pressure for resistance, for example, in discarded peel from citrus fruit that has previously been treated.

A total 30 agricultural azoles have been authorized for agricultural use in the past 20 years, of which five agricultural azoles showed the potential strong selection for resistance (Snelders et al. 2012). In our experiment, we confirmed that these agricultural azoles can induce cross-resistance to medical azoles. Of these five azoles, difenoconazole exposed strongest selection. Thus, the use of similar compounds for medical and non-medical applications may result in the selection of resistance through one application that affects the use of similar compounds in the other area of use. Cross-resistance has been found in other applications as well, for instance, for extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli*, where the use of antibacterial agents for cattle rearing has selected for antibiotic resistance. The resistant bacteria are transferred to humans through consumption of food containing ESBL *E. coli*, which may then cause infections in humans that are difficult to treat due to cross-resistance to medical drugs (dos Santos et al. 2013).

Mutation is the driving force for azole resistance evolution. Population dynamic characterization showed that the colony size (big and small), the colony texture (dense and fluffy), and even sporulation (early and late) changed over the evolutionary experiment in the different lineages, which suggested the fungi adapt to the azole environment through different genetic changes. These mutated genes which may relate with growth, sporulation need further study. Interestingly, the sporulation defect mutant in d6 lineage had a high resistance level which could be explained by the trade-off between hyphal growth and resistance traits (Mille-Lindblom et al. 2006). Previous work has shown that mutations in the *cyp51A* gene of the ergosterol synthesis pathway can infer resistance to both the agricultural and the medical azoles used, due to the similarities in chemical structure (Snelders et al. 2012; Gisi 2013). With our whole-genome sequencing, we detected mutations in the *cyp51A* gene, as well as mutations in other targets. It is known that other mutations than mutations in the *cyp51A* gene can confer cross-resistance to medical azoles (Camps et al. 2012a). In D6 white, a

hypothetical protein mutation is associated with resistance, and needs further gene identification. D3 dense and d1 middle contain a mutation in HMG CoA reductase, a rate-controlling enzyme of the mevalonate *pathway*, that produces ergostrol. In d3 fluffy and d6 fluffy with variable SNP, are difficult to trace back from evolutionary experiment. This might be explained by sampling error since only three colonies were used for sequencing. In d6 fluffy, Mdm31 is an inner membrane protein of the mitochondria, while Tom70 is located in the outer membrane. Both are in the protein influx pathway of the mitochondria(Bolender et al. 2008) It seems that the mitochondrial dysfunction has been associated with azole resistance. However, the mechanism by which the mutations found in these two phenotypes confer resistance needs, however, to be further studied.

Our results demonstrated population-dynamic changes (colony size, texture, sporulation, and cyp51A mutation frequency) that are associated with increased azole resistance in *A. fumigatus* over seven-week experimental evolution. As we observed differences in selection pressure for the various azole compounds, the implications of both the dose and the molecule structure should be taken into account in future studies. Knowing the exact identity of adaptive mutations in *A. fumigatus* will be an essential avenue of future research, which will further unravel the evolutionary dynamics of *A. fumigatus* adaptation both in the human setting and in the environment. Understanding the key factors that facilitate resistance selection in *A. fumigatus* is essential to design strategies that prevent or overcome this emerging threat.

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Supplementary data

Supplementary 1A

Results of an Analysis of Variance (ANOVA) for the relative MIC data with type of Azole (Azole) and Resistance to three medical azoles (Test) as fixed factors (Relative MIC ~ azole treatment*medical azoles test; *: *P*-values lower than 0.05 **: *P*-values lower than 0.01).

Source of variation	Sum of squares (*SS*)	Degrees of freedom (*df*)	Mean squares (*MS*)	*F*	Significance (*P*-value)
Azole (A)	2248.333	5	449.667	4.074	0.002**
Test (T)	3499.056	2	1749.528	15.852	0.000**
A*T	3207.611	10	320.761	2.906	0.003**
Error	9933.000	90	110.367		
Total	23688.000	108			

Supplementary 1B

Following Post Hoc LSD Tests to explain which medical azole test has significantly effect on the relative MIC. (Relative MIC ~ medical azoles test; Post Hoc LSD Tests *: *P*-values lower than 0.05 **: *P*-values lower than 0.01)

P (Post Hoc)	MIC(Itraconazole)	MIC(voriconazole)	MIC(posaconazole)
MIC(Itraconazole)		P <0.01**	P <0.01**
MIC(voriconazole)	P <0.01**		P = 0.679
MIC(posaconazole)	P <0.01**	P = 0.679	

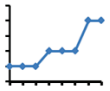
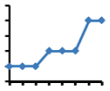
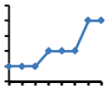
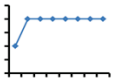
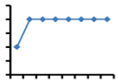
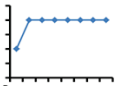
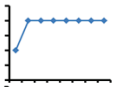
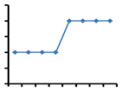
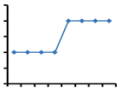
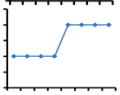
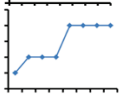
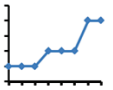
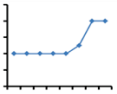
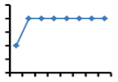
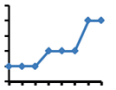
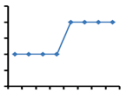
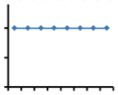
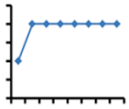
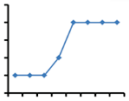
Supplementary 1C

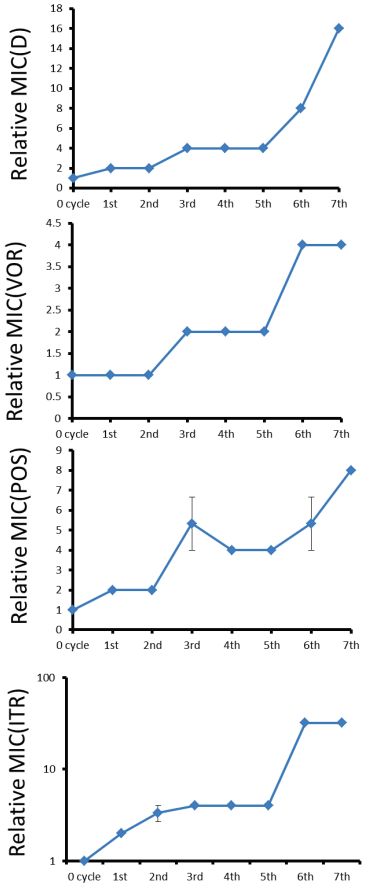
Following Post Hoc LSD Tests to explain which agricultural azole has significantly effect on the cross resistance to medical azoles. (Relative MIC ~azoles; Post Hoc LSD Tests *: *P*-values lower than 0.05 **: *P*-values lower than 0.01)

Azole \ *P*	Bromuco n-azole	Difenocon -azole	Epoxicon-azole	Propico n-azole	Tebucon-azole	Control (no azole)
bromuconazole		0.002**	0.42	0.67	0.97	0.94
difenoconazole	0.002**		0.016**	0.006**	0.002**	0.00**
epoxiconazole	0.42	0.016**		0.704	0.439	0.516
propiconazole	0.67	0.006**	0.704		0.693	0.757

tebuconazole	0.97	0.002**	0.439	0.693		0.932
Control(no azole)	0.94	0.001**	0.516	0.757	0.932	

Supplementary 2A

Evolutionary trajectory pattern	Pattern of agricultural azole	Pattern of POS	Pattern of VOR	Pattern of ITR
D1, D3, D6, E4, P3				
D4, B6, T4, E1				
B5				
T1				
P2				



Supplementary 2B

Linear regression of analysis of correction between MIC trajectory of agricultural azole and MIC trajectory of three medical azoles (POS, VOR, ITR)

Regression	**D1(POS)**	**D1(VOR)**	**D1(ITR)**
D1(D)	R=0.796, $F_{1,22}$ = 37.983, P <0.01**	R=0.873, $F_{1,22}$ = 70.328, P <0.01**	R=0.876, $F_{1,22}$ = 72.849, P <0.01**
Regression	**D4(POS)**	**D4(VOR)**	**D4(ITR)**
D4(D)	R=0.664, $F_{1,22}$ = 17.320, P <0.01**	R=1.00, $F_{1,22}$ = -,P <0.01**	R=0.646, $F_{1,22}$ = 15.775, P <0.01**
Regression	**D5(POS)**	**D5(VOR)**	**D5(ITR)**
D5(D)	R=0.624, $F_{1,22}$ = 14.007, P <0.01**	R=0.701, $F_{1,22}$ =21.218,P <0.01**	R=0.796, $F_{1,22}$ = 38.060, P <0.01**
Regression	**D6(POS)**	**D6(VOR)**	**D6(ITR)**
D6(D)	R=0.864, $F_{1,22}$ = 64.809, P <0.01**	R=0.805, $F_{1,22}$ =40.525,P <0.01**	R=0.836, $F_{1,22}$ = 51.118, P <0.01**
Regression	**T1(POS)**	**T1(VOR)**	**T1(ITR)**
T1(T)	R=0.525, $F_{1,22}$ = 8.361, P <0.01**	R=0.659, $F_{1,22}$ =16.844 P <0.01**	R=0.650, $F_{1,22}$ = 16.058, P <0.01**
Regression	**T4(POS)**	**T4(VOR)**	**T4(ITR)**
T4(T)	R=0.974, $F_{1,22}$ = 412.5, P <0.01**	R=0.808, $F_{1,22}$ =41.365 P <0.01**	R=0.934, $F_{1,22}$ = 149.904, P <0.01**
Regression	**B5(POS)**	**B5(VOR)**	**B5(ITR)**
B5(B)	R=0.444, $F_{1,22}$ = 5.392, P <0.05*	R=1.00, $F_{1,22}$ =-, P <0.01**	R=0.965, $F_{1,22}$ = 297, P <0.01**
Regression	**B6(POS)**	**B6(VOR)**	**B6(ITR)**
B6(B)	R=1.00, $F_{1,22}$ = -, P <0.01**	R=0.378, $F_{1,22}$ =3.667, P =0.069	R=0.608, $F_{1,22}$ = 12.891, P <0.01**
Regression	**E1(POS)**	**E1(VOR)**	**E1(ITR)**
E1(E)	R=0.618, $F_{1,22}$ = 13.606, P <0.01**	R=0.714, $F_{1,22}$ =22.846, P <0.01**	R=0.799, $F_{1,22}$ = 38.717, P <0.01**
Regression	**E4(POS)**	**E4(VOR)**	**E4(ITR)**
E4(E)	R=0.836, $F_{1,22}$ = 50.869, P <0.01**	R=0.832, $F_{1,22}$ =49.417, P <0.01**	R=0.863, $F_{1,22}$ = 64.095, P <0.01**
Regression	**P3(POS)**	**P3(VOR)**	**P3(ITR)**
P3(P)	R=0.825, $F_{1,22}$ = 47.028, P<0.01**	R=0.920, $F_{1,22}$ = 121.352, P<0.01**	R=0.74, $F_{1,22}$ = 26.666, P<0.01**
Regression	**P2(POS)**	**P2(VOR)**	**P2(ITR)**
P2(P)	R=0.079, $F_{1,22}$ =0.138, P=0.714	--	R=0.176 $F_{1,22}$ = 0.7, P=0.412

Supplementary 3A

One –way ANOVA followed Post Hoc analysis of mycelial growth rate of evolved isolates and ancestor to show the difference between the evolved isolates and ancestor

Statistic / **Evolved types**	**Ancestor**
d1-middle	$F_{2,6}$= 1463.769, P <0.01, Post-Hoc LSD test, $P_{ancestor\text{-}middle}$ <0.01**
d1-big	$F_{2,6}$= 1463.769, P <0.01, Post-Hoc LSD test, $P_{ancestor\text{-}big}$ <0.01**
d3-dense	$F_{2,6}$= 10299, P<0.01, Post-Hoc LSD test, $P_{ancestor\ –dense}$ <0.01**
d3-fluffy	$F_{2,6}$= 10299, P<0.01, Post-Hoc LSD test, $P_{ancestor\ -fluffy}$ <0.01**
d6-big	$F_{3,8}$= 1771.202, P<0.01, Post-Hoc LSD test, $P_{ancestor\text{-}big}$ <0.01**
d6-fluffy	$F_{3,8}$= 1771.202, P<0.01, Post-Hoc LSD test, $P_{ancestor\text{-}fluffy}$ <0.01**
d6-white	$F_{3,8}$= 1771.202, P<0.01, Post-Hoc LSD test, $P_{ancestor\text{-}white}$ =0.103

Supplementary 3B

One –way ANOVA followed Post Hoc analysis of spore production of evolved isolates and ancestor to show the difference between the evolved isolates and ancestor

Statistic / **Evolved types**	**Ancestor**
d1-middle	$F_{2,6}$= 69.095, P <0.01, Post-Hoc LSD test, $P_{ancestor\text{-}\ middle}$ = 0.891
d1-big	$F_{2,6}$= 69.095, P <0.01, Post-Hoc LSD test, $P_{ancestor\text{-}big}$ <0.01**
d3-dense	$F_{2,6}$= 586.204, P <0.01, Post-Hoc LSD test, $P_{ancestor\text{-}dense}$ <0.01**
d3-fluffy	$F_{2,6}$= 586.204, P <0.01, Post-Hoc LSD test, $P_{ancestor\text{-}fluffy}$ <0.05*
d6-big	$F_{3,8}$= 771.498, P<0.01, Post-Hoc LSD test, $P_{ancestor\text{-}big}$ <0.05*
d6-fluffy	$F_{3,8}$= 771.498, P<0.01, Post-Hoc LSD test, $P_{ancestor\text{-}fluffy}$ <0.01**;
d6-white	$F_{3,8}$= 771.498, P<0.01, Post-Hoc LSD test, $P_{ancestor\text{-}white}$ <0.01**

Supplementary 4. SNPs calling between the ancestor and evolved isolates with differnet morphotypes.

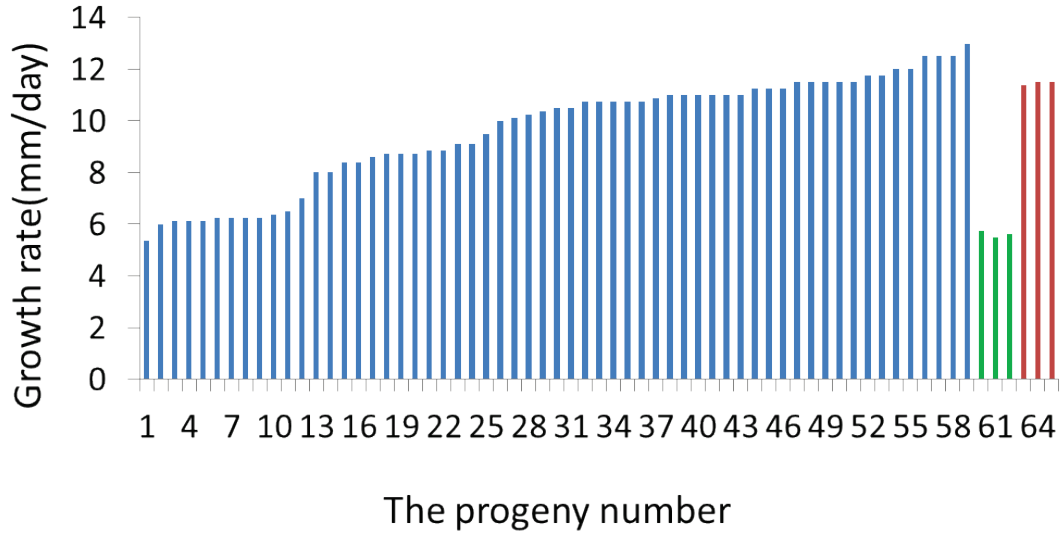
Ref chroms-ome (Clinical reference strainAF293)	New genome (de novo assembly)	Gene Position	Snp	anc	Con2	Con4	Con5	D1big	D3den	D3fluffy	D6fluffy	D6white
4 / 1784849	3031 / 138096	cytochrome P450 14-alpha sterol demethylase [Aspergillus fumigatus] Sequence ID: gb|AKE50937.1|	C -> T 138 G->S	C G 54	. . 41	. . 44	. . 60	T S 53	. . 45	. . 42	. . 59	. . 47
2 / 986005	3218 / 54189	HMG-CoA reductase [Aspergillus fumigatus Z5] Sequence ID: gb|KMK57733.1|	C -> T 320 P -> L	C P 40	. . 39	. . 54	T L 52	T L 48	T L 40	. . 38	. . 34	. . 48
2 / 3435913	3150 / 226430	Putative amidohydrolase ytcJ [Aspergillus fumigatus Z5] Sequence ID: gb|KMK61702.1|Or conserved hypothetical protein [Aspergillus fumigatus Af293] Sequence ID: ref|XP_755663.1|	C -> T 374 P -> S	C P 40	C / T P / S 41 / 4	. . 34	. . 34	. . 36	. . 51	C / T P / S 7 / 21	. . 40	. . 41
3 / 568217	3074 / 407507	alpha,alpha trehalose glucohydrolase TreA/Ath1 [Aspergillus fumigatus var. RP-2014] Sequence ID: gb|KEY75594.1|	C -> T 109 S -> L	C S 45	. . 37	. . 39	. . 52	. . 43	. . 46	C / T S / L 11 / 49	. . 54	. . 45
	3144 / 36219	Conserved hypothetical protein [Aspergillus funmigates Af293] Sequence ID: ref|XP_749671.1|	TG -> (-) Frameshift and stop codon*	TG H			(-) Frameshift					
5 / 2390677	3134 / 161643	DUF1479 domain protein [Aspergillus fumigatus Af293] Sequence ID: ref|XP_753711.1|	G -> A 276 A -> T	G A 50	. . 45	. . 39	. . 44	. . 50	. . 40	G / A A / T 19 / 34	. . 47	. . 47

Ref chroms-ome (Clinical reference strainAF293)	New genome (de novo assembly)	Gene Position	Snp	anc	Con2	Con4	Con5	D1big	D3den	D3fluffy	D6fluffy	D6white
	3178 78573	PtaB protein [Aspergillus fumigatus Af293] Sequence ID: ref|XP_755622.1|	C -> T 264 Q -> STOP	C Q 35	. . 39	. . 37	. . 43	. . 38	. . 29	. . 30	. . 34	T STOP 31
7/ 1837636	3183 30261	hypothetical protein AFUB_092560 [Aspergillus fumigatus A1163] Sequence ID: gb|EDP48538.1|	C -> T 167 G -> D	C G 58	. . 44	. . 38	. . 53	. . 66	. . 42	. . 60	. . 58	T D 48
	3120 13282	U3 small nucleolar RNA-associated protein Utp11 [Aspergillus fumigatus Af293] Sequence ID: ref|XP_752827.1|	C ->G 199 D -> E	C D 39	C /G D / E 23 / 24	. . 44	. . 39	. . 37	. . 40	. . 39	. . 54	. . 38
	3120 15850	phosphoribosyl-AMP cyclohydrolase [Aspergillus fumigatus Af293] Sequence ID: ref|XP_752826.1|	C -> A 325 W-> C	C W 39	C /A W / C 16 / 15	. . 40	. . 40	. . 48	. . 38	. . 45	. . 37	. . 41
	3084	mitochondrial outer membrane translocase receptor (TOM70), putative [Aspergillus fumigatus Af293] Sequence ID: ref|XP_749301.1|	C -> T 107 Q -> STOP	C Q 43	. . 55	. . 49	. . 37	. . 34	. . 56	. . 51	C / T Q / STOP 25 / 13	. . 46
	3120 21378	Mn superoxide dismutase MnSOD [Aspergillus fumigatus Af293] Sequence ID: ref|XP_752824.1|	C -> A 129 G -> V	C G 60	. . 36	. . 39	. . 46	. . 53	. . 52	. . 42	C / A G / V 40 / 6	. . 58

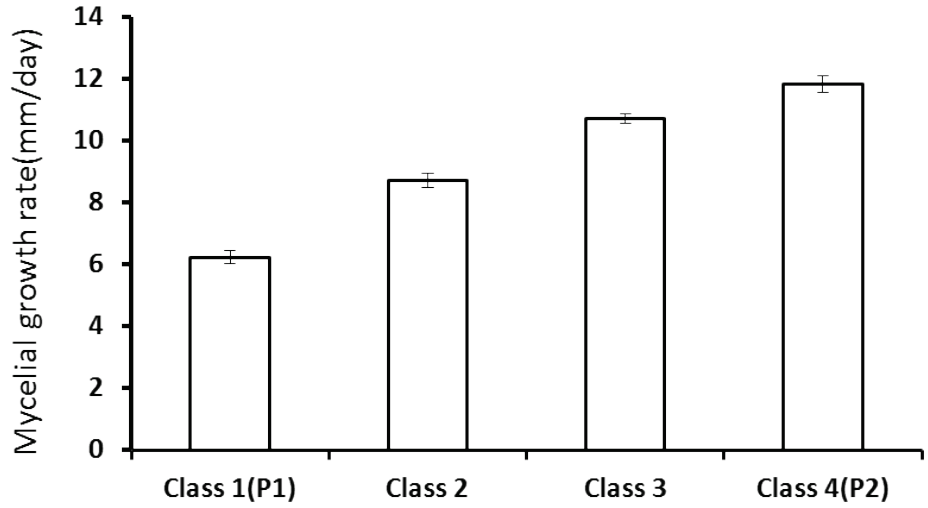
*From amino acid 551 **HRTVIISP to QNCYHFA(STOP)**

Supplementary Figure 5

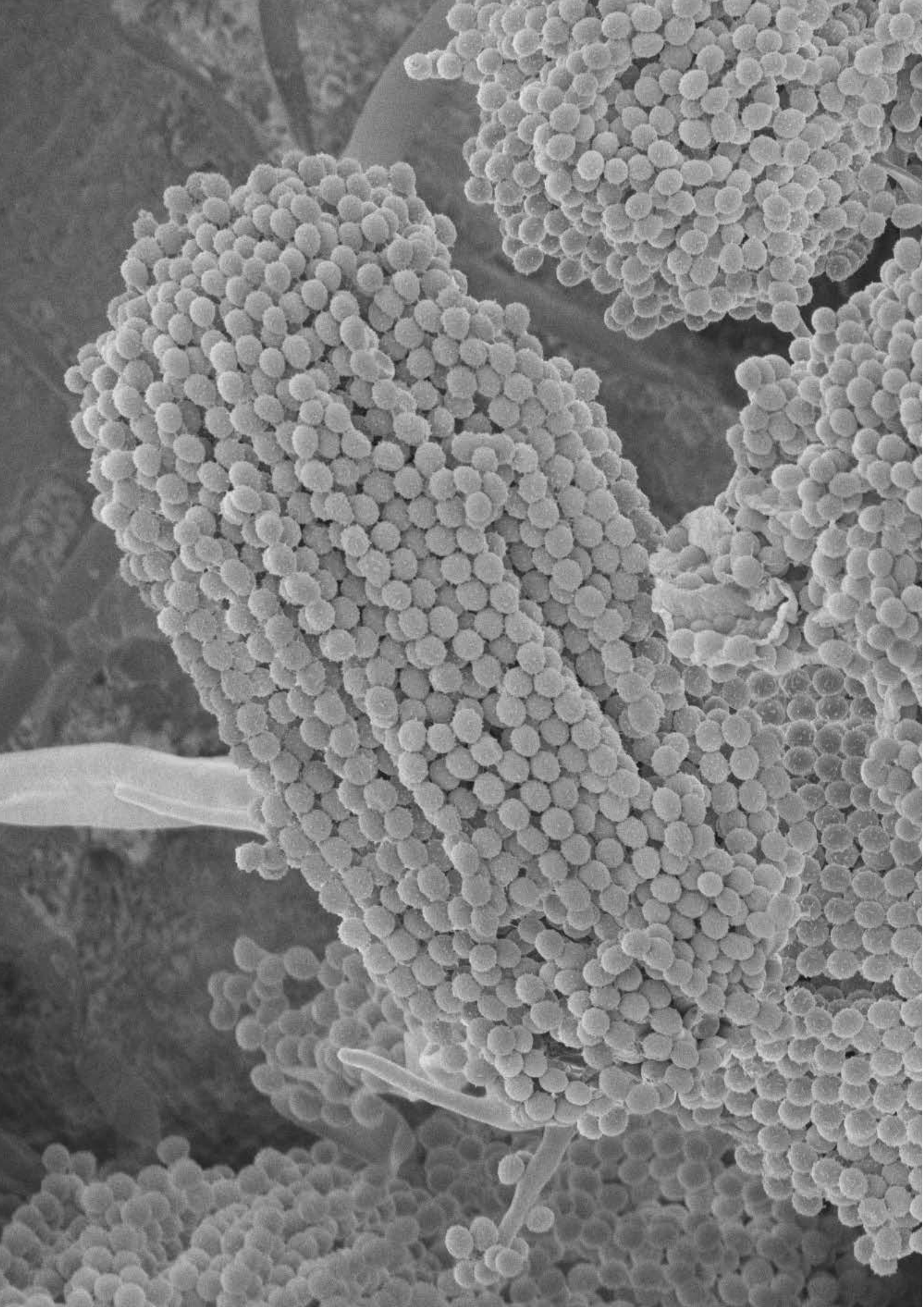
Analysis of the progeny of a sexual cross between D1big and sensitive isolate. The four phenotypic classes suggest two fixed mutations which is in line with the observed two-step increase in resistance ("Growth rate") during the evolutionary trajectory.



1-59 are progeny number; 60-62: parental type 1, ancestor of the evolution experiment (green); 63-65: parental type 2, evolved strainD1big (red)



S5. Segregation of MGR among the progeny from a sexual cross between the ancestral genotype (parental type 1; P1) and an evolved strain (D1big, parental type 2; P2) with high MIC and fitness. MGR of progenies was measured on the MEA containing 1 µg /mL difenoconazole. The *top panel* shows all progeny separately and the two parental types for the cross, the *bottom panel* shows how the progeny clusters in distinct phenotypic classes (evenly classify the group till ANOVA and following post hoc significant test shown). Among the progeny we clearly observe segregation in 4 classes, two parental classes and two recombinant classes. This is indicative of two segregation loci, which in turn suggests that two mutations with an effect on MGR had fixed during experimental evolution.



CHAPTER V

Relevance of heterokaryosis for adaptation and azole-resistance development in *Aspergillus fumigatus*

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Abstract

Aspergillus fumigatus causes a range of diseases in humans, some of which are characterized by fungal persistence for many years. *A. fumigatus,* being a generalist saprotroph, may initially establish lung colonisation by its physiological versatility and subsequently gradually adapt to the human lung environment, through genetic changes. Human-adapted genotypes can be generated by spontaneous mutation and/or recombination and subsequent selection of the fittest genotypes. Sexual and asexual spore formations are considered to be crucial contributors to the genetic diversity and adaptation potential of aspergillus. However, in certain aspergillus diseases, such as cystic fibrosis, *A. fumigatus* may persist as network of mycelium and develop patient-acquired resistance during azole therapy. Such mycelia may become heterokaryotic by mutation of one of the nuclei or by anastomosis of hyphae from genetically different mycelia. We investigated the relevance of heterokaryosis in the adaptive development of azole resistance in *A. fumigatus.* We found that azole-exposed mycelial cultured from patients, as well as from laboratory evolution experiments, segregated into different morphotypes with different azole-resistance phenotypes. Individual colonies recovered from the same patient were capable of forming a heterokaryon, whereas those from different patients were heterokaryon-incompatible. We formed heterokaryons and diploids from *A. fumigatus* strains with different levels of resistance. When exposed to various azole environments, the heterokaryons revealed remarkable shifts in the nuclear ratio, and the resistance level of most heterokaryons exceeded that of the corresponding heterozygous diploids. Our results indicate that heterokaryosis may be a strategy for *A. fumigatus* to adapt to the lung environment, field and to overcome azole exposure.

Key words: *Aspergillus fumigatus*; heterokaryon; azole resistance; diploid; flexible; nuclear ratio;

Introduction

The vast majority of invasive mould infections in humans are caused by *Aspergillus fumigatus*. Azole antifungals are the mainstay of management of aspergillus diseases, but treatment is hampered by the emergence of multi-azole resistant *A. fumigatus* isolates. Azole resistance is now reported globally (Arendrup et al. 2010; Lockhart et al. 2011; Mortensen et al. 2011; Pfaller et al. 2011; Chowdhary et al. 2012; Morio et al. 2012; Gisi 2013; Bignell 2014; Chowdhary et al. 2014), with resistance rates varying between 3.9% and 19% in clinical isolates in the Netherlands (Snelders et al. 2008; van der Linden et al. 2011) and 20% in the National Aspergillosis Centre of the United Kingdom (Howard et al. 2006; Howard et al. 2009; Bueid et al. 2010). The majority of highly resistant mutants have modifications in the coding or promoter region of the *cyp51A*-gene. This gene encodes the enzyme lanosterol 14- alpha-demethylase (CYP51), the target of azoles that is essential to the ergosterol synthesis pathway. These *cyp51A* mutations are believed to emerge by exposure of *A. fumigatus* to medical azoles or azole fungicides (Camps et al. 2012).

Although many aspects of azole-resistance development in *A. fumigatus* remain to be investigated, resistance can be regarded as a fungal adaptation strategy to azole-containing environments, through creating genetic diversity followed by natural selection (McDonald et al. 2016). Genetic variation may be generated during the various aspects of the lifecycle of *A. fumigatus*: mycelial growth, asexual sporulation and sexual reproduction. Several studies in *A. fumigatus* have focused on the consequences of asexual (Zhang et al. 2015b) and sexual reproduction for fungal adaptation (Kwon-Chung and Sugui 2009; Losada et al. 2015), but the role of mycelial growth has been largely overlooked. Although patient-acquired azole resistance appears to be associated with the presence of a cavity, which is an environment that allows asexual reproduction, *A. fumigatus* hyphal biofilms may be present in patients with cystic fibrosis (CF). A variety of azole-resistance mutations has been observed in CF-patients, indicating that fungal adaptation takes place (Arendrup et al. 2010). Somatic mutations may occur during mitotic divisions leading to genetic variation within the mycelium. Such heterokaryotic mycelium may subsequently undergo parasexual recombination and/or segregate a variety of clones.

Heterokaryosis, i.e. genetically different nuclei within the same cytoplasm, is common in fungi. Hansen (1938) found that in various fungi more than 50% of natural mycelia were heterokaryons that upon single-spore culturing segregated in three morphologically distinct types of pure cultures: one that produces scant aerial hyphae but many conidia, one that produces abundant aerial hyphae but few or no conidia, and an intermediate type (Hansen 1938).

A heterokaryon can result from mutations in one or more nuclei in a homokaryotic mycelium, or from anastomosis of hyphae from genetically distinct homokaryons. The

latter is however restricted by heterokaryon incompatibility, a common fungal allorecognition mechanism limiting successful fusion of hyphae to clonally related strains with the same heterokaryon compatibility alleles (Glass and Kuldau 1992; Saupe 2000; Bastiaans et al. 2014; Bastiaans et al. 2015), so mutation is probably a more likely initial cause of heterokaryon formation, especially for isolated long-lasting cultures.

Like heterozygosity, heterokaryosis may allow genetic complementation of recessive mutations and heterosis effects. However, heterokaryons are potentially more flexible: in a heterokaryon the nuclear (and allelic) ratio is not strictly controlled, so all possible nuclear ratios can occur from 0 to 100 percent and can change depending on environmental conditions, whereas in a heterozygous diploid the allele ratio is fixed at 50 percent. Heterokaryosis may also lower the fitness of the colony because of genomic conflict between different types of nuclei. Indeed cheater nuclei, that increase their frequency among spores at the cost of overall colony fitness, and are poorly sporulating when homokaryotic, have been found to evolve rapidly under specific conditions in *Neurospora crassa* that allow heterokaryon growth (Bastiaans et al. 2014). These lab findings are reminiscent of the natural heterokaryons by Hansen (1938). Even though heterokaryosis has been described in many fungal species such as *Neurospora, Aspergillus*, including *A. fumigatus,* and *Penicillium*(Dodge 1942; Beadle and Coonradt 1944; Pontecorvo and Gemmell 1944; Jinks 1952; Debets et al. 1990), the biological significance is largely unclear (Debets 1998).

Since asexual spores of *A. fumigatus* are uninucleate, newly formed colonies start as a homokaryon and may produce heterokaryon during mycelial growth. Heterokaryosis is thus a transient characteristic of the mycelium that is lost upon asexual sporulation and dispersal of spores by air or by segregation into homokaryotic sectors. Therefore, heterokaryons may form and persist particularly in long-lived cultures. This may be the case for chronic *A. fumigatus* infections, where the fungus has been shown to persist sometimes for many years (De Valk et al. 2009). Moreover, in patients who have been treated with triazoles, heterokaryosis might be a strategy to develop resistance (Camps et al. 2012a). Over time, emergence of azole resistance in chronically colonized patients has indeed been described in consecutive *A. fumigatus* cultures that were concluded to be isogenic using microsatellite genotyping (Arendrup et al. 2010; Camps et al. 2012b). Evidence for a possible role of heterokaryosis in azole-resistance development was found in evolutionary laboratory experiments where an ancestral strain was allowed to evolve resistance during several weeks of azole exposure (Zhang et al. 2015b). Both from patients and from evolutionary laboratory experiments, different evolved morphological clones of *A. fumigatus* have been isolated, some of which are poorly sporulating or completely aconidial (Ahmad et al. 2015; Zhang et al. 2015a), Table 1 and Figure 1). Different morphotypes have been encountered especially in chronic infections either sinusitis, aspergilloma or in CF (De Valk et al. 2007; de Valk et al. 2009; Mortensen et al. 2011; Ahmad et al. 2015; Zhang et al. 2015a) but the significance of such polymorphisms has not been studied.

Here, we study the relevance of heterokaryosis and morphotypes in the development of azole-resistance in *A. fumigatus.* We address the following questions.

a. What are the characteristics of different morphotypes of *A. fumigatus* isolated from evolved cultures of patients or lab experiments?
b. Are the different successive or coexisting morphotypes heterokaryon–compatible as expected for isogenic isolates?
c. What is the azole resistance of heterokaryons relative to that of the individual homokaryon and heterozygous diploids?
d. Is there plasticity in the nuclear ratio within a heterokaryon in response to changing azole concentrations?

Material and methods

Twelve clinical *A. fumigatus* strains, from four aspergillosis patients from Radboud University Medical Centre, were available (Arendrup et al. 2010; Camps et al. 2012b). *A. fumigatus* CBS 140053 was isolated from an environmental field in Wageningen, The Netherlands in 1992. CBS 140053 -D_1-7#, d_3-7# and d_6-7# are the evolved strains from a seven-week evolution experiment under the exposure of difenoconazole (1 μg/mL) from which also d_3-7#-dense, d_3-7#-fluffy, d_6-7#white and d_6-7#-fluffy stains were collected (Zhang et al. 2015b). The azole fungicide difenoconazole, and three medical azoles (itraconazole (ITR), voriconazole (VOR) and posaconazole (POS)) were purchased from Sigma Company (Sigma Aldrich, Germany) (Zhang et al. 2015b). The number and spore size of *A. fumigatus* were measured on a Coulter counter (Beckman Coulter, the Netherlands).

Culture media

Minimal Medium (MM) was used for culturing the heterokaryons and heterozygous diploids. MM consists of 6.0 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g $MgSO_4$7. $7H_2O$, 0.5 g KCl, 10 mg of $FeSO_4$, $ZnSO_4$, $MnCl_2$ and $CuSO_4$ and agar 15 g + 1000 mL H_2O (pH 5.8). Malt extract ager was used for counting spores and measuring of growth rate, purchased from Sigma Company (Sigma Aldrich, Germany).

Different morphotypes of *A. fumigatus* isolated from evolved cultures of patients and experimental evolution experiments

The strains used in this study are listed in Table 1. From each of four patients, in total twelve clinical isolates were obtained (Arendrup et al. 2010; Camps et al. 2012b). Four successive isolates were collected from the same tissue from patients 1 (aspergilloma) and 2 (chronic granulomatous disease) during treatment, another four strains were from patient 3, a kidney transplant recipient, and patient 4 with invasive aspergillosis. From an evolutionary experiment the ancestral strain CBS140053 and five derived

strains evolved during seven weeks on medium with 1 µg/mL difenoconazole were used (Zhang et al. 2015b; Table 1).

Heterokaryon compatibility testing

Heterokaryon compatibility of strains was tested following standard methods (Cove 1976; Debets et al. 1990; Todd et al. 2007). Recessive markers (nitrate non-utilizing mutations *nia* and *cnx* and a colour mutation) were introduced in the various strains and strains with complementing *nia* and *cnx* markers were co-inoculated on medium with nitrate as the sole N-source (Supplement 1). The effects of chlorate and colour makers on the growth rate and ratio changes in the heterokaryon are provided in Supplement 2. As a control these strains were grown as homokaryons on the same media. Heterokaryons of compatible complementing strains show typical vigorous growth after seven days. Heterozygous diploids were isolated from heterokaryons by means of the sandwich method (Bos et al. 1988).

Azole resistance assays

MGR assays were previously shown to correlate well with MIC assays (Zhang et al. 2015) and were performed as follows by averaging the colony diameters (in mm) as measured in two randomly chosen perpendicular directions after four days of growth colony. Heterozygous diploids and constructed heterokaryons were inoculated on MM medium and MM medium with 1µg/ml of difenoconazole.

The nuclear ratio in heterokaryons in different azole environments

Assuming that the nuclear ratio among the conidiospores reflects that nuclear ratio within the mycelium, we tested the effect of azole on the constitution of the heterokaryon. Briefly, heterokaryons, from sensitive and resistant strains differing in conidial colour, *nia* or *cnx* mutation, S (susceptible)&S(CBS-140053& CBS-140053), I (intermediate) &S (D3-7#-dense&CBS-140053) and R (resistant) &S (D1-7#&CBS-140053) were grown on MM plates with or without 1 ug/mL difenoconazole. After 5 days of growth, spores were harvested from the edge of the heterokaryon into 0.5 mL of saline (distilled water with NaCl 0.8 g/L) supplemented with Tween 80 (0.05 % v/v) and dilutions were spread on a MEA plate. The number of colonies of either colour was counted and the ratio was calculated. T-test was used for analyzing the significance of the nuclear shift.

Results

All isolates with distinct morphology from the same patient or the same evolution experiment are heterokaryon compatible.

The four *A. fumigatus* strains from patient 1 and four of patient 2, which were sampled in succession, showed an increase in spore size, a decrease in growth rate and an increase in azole resistance (Table 1, Figure 1). The two isolates of patients 3 and the two of patient 4, isolated from the same clinical sample, were morphologically very distinct (Figure 1). Also the cultures collected in the seven-week evolution experiment showed different morphology when compared to the ancestor and each other in addition to increasing azole resistance (Table 1, Figure 1).

Isolates from the evolution experiments all share a common ancestor and are therefore expected to be heterokaryon compatible. Indeed all isolates were heterokaryon compatible, showing vigorous growth on nitrate medium between isolates with complementing nitrate-nonutilizing mutations (Figure 1, E1, 3, 6 A, column). This indicates a stable heterokaryon compatibility. Also the four isolates from patients 1 and 2 and the two from patient 3 were heterokaryon compatible (examples are shown in Figure 1, P1-3, A column). It was not possible to obtain nitrate-nonutilizing mutants from the non-sporulating isolate from patient 4 and therefore did not allow for heterokaryon testing. Isolates from different patients and lab experiments all showed heterokaryon incompatibility as expected from non-isogenic isolates (Figure 1, B column).

Table 1. Successive and co-existing isolates from four patients and from laboratory evolution experiments with different macroscopic morphology, azole resistance mutations and resistance phenotype. Note: S: sensitive; I: intermediate; R: resistant; itraconazole (ITR), voriconazole (VOR) and posaconazole (POS)

		Strains	time	Morphs									
				Spore size (µm)/colony structure	Growth rate (see Figure 1)	Virulence (camp2012)	Mutation on cyp51	Resistance Class	Specimen	Treatment	MIC ITC	VOR	POS
Clinical	Patient 1	V74-61	29-9-2008	2.55 ± 0.02	9.375 ± 0.03	--	A9T	Sensitive (S)	sputum	ITC	0.5	1	0.063
		V77-41	17-12-2008	2.65 ± 0.01	8.125 ± 0.02	--	A9T,F291I	Resistant(R)	sputum	POS	>16	1	1
		V80-28	9-3-2009	2.65 ± 0.01	6.875 ± 0.01	--	A9T,F291I	Resistant(R)	sputum	POS	>16	8	>16
		V83-14	7-6-2009	2.70 ± 0.01	6.625 ± 0.01	--	A9T,G54E	Resistant(R)	bal	L + AMB + CAS	>16	0.5	1
	Patient 2	V67-35	0 week	2.50±0.02	10.25 ± 0.04		--	Sensitive (S)	respiratory samples	Voriconazole	0.125 /0.5	0.5/ 1.0	0.016/ 0.125
		V67-36	108weeks	2.72±0.01	8.875 ± 0.02		--	Sensitive (S)	respiratory samples	Caspofungin + posaconazole	0.25/ 0.5	0.5/ 1	0.031/ 0.125
		V67-37	125weeks	2.87±0.02	8.375 ± 0.03		--	Resistant(R)	respiratory samples	Caspofungin + posaconazole	>16/> 4	4/>4	0.25/0. 5
		V67-38	127 weeks	2.87±0.02	6.25 ± 0.01		--	Resistant(R)	respiratory samples	Caspofungin + posaconazole	>16/> 4	4/>4	0.25/1
	Patient 3	V094-15	20-2-2010	Fluffy/small	4.125 ± 0.02	--	TR46	Resistant(R)	subcutaneous abscess	?	1	>16	0.25
		V094-16	20-2-2010	big	7.375 ± 0.01	--	TR46	Resistant(R)	subcutaneous abscess	?	0.5	>16	0.25
	Patient 4	V186-81	03-09-2015	Green sporulation	6.625 ± 0.01	--	--	Sensitive(S)	sfenoid		-	-	-
		V187-02	03-09-2015	White non-sporulation	3.75 ± 0.02	--	--	Sensitive(S)	sfenoid				
Laboratory		CBS 140053	0 week	Normal	3.125 ± 0.01	--	--	Sensitive(S)	Agricultural filed	--	0.125	0.5	0.016
	E1	CBS 140053 D_1-7#	7 weeks	Big size	8.625 ± 0.03	--	G138S	Resistant(R)	Evolutionry lines	Difenoconazole	32	4	0.5
	E3	CBS 140053 D_3-7#	7weeks	Fluffy	6.25 ± 0.03	--	--	Intermediate (I)	Evolutionry lines	Difenoconazole	8	2	0.5
		CBS 140053 D_3-7#	7weeks	Dense	6.125 ± 0.01	--	--	Intermediate (I)	Evolutionry lines	Difenoconazole	8	1	0.5
	E6	CBS 140053 D_6-7#	7 weeks	white	3.125 ± 0.02	--	--	Intermediate (I)	Evolutionry lines	Difenoconazole	8/16	2	0.25
		CBS 140053 D_6-7#	7 weeks	fluffy	6.25 ± 0.01	--	--	Intermediate (I)	Evolutionry lines	Difenoconazole	8	2	0.5

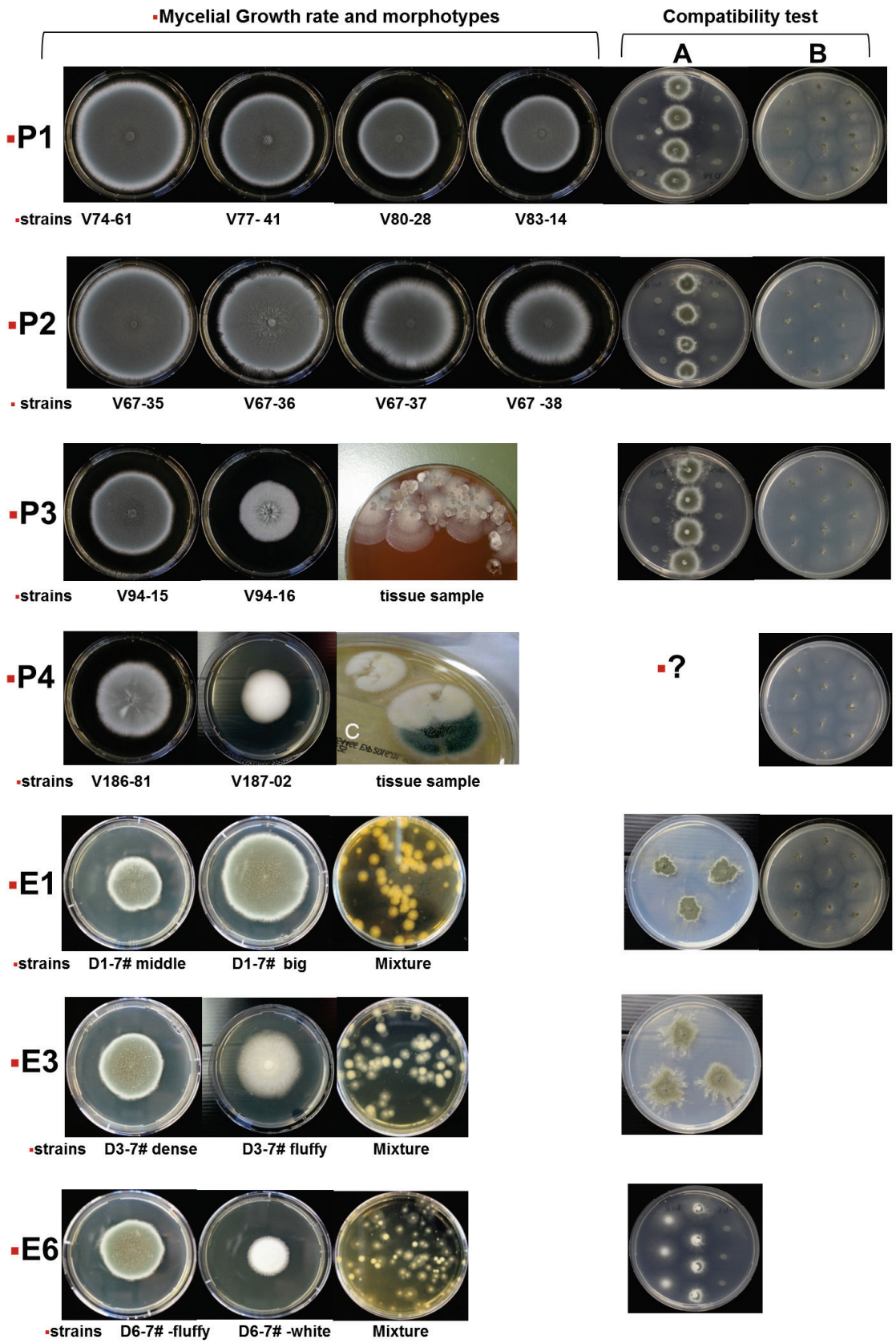


Figure 1. Compatibility–test of all clinical strains and evolutionary strains

A; compatibility test between the isolates from the same patients or lab condition

B; compatibility test between isolates from different patients and lab condition

A. fumigatus heterokaryons have higher azole resistance than heterozygous diploids

The azole resistance level, measured as the mycelial growth rate on azole-containing medium, of heterozygous diploids was compared to that of heterokaryons and their constituting haploid strains (Figure 2). The azole resistance of the heterokaryons was significantly higher than of the heterozygous diploids, but lower than of the most resistant haploid strain.

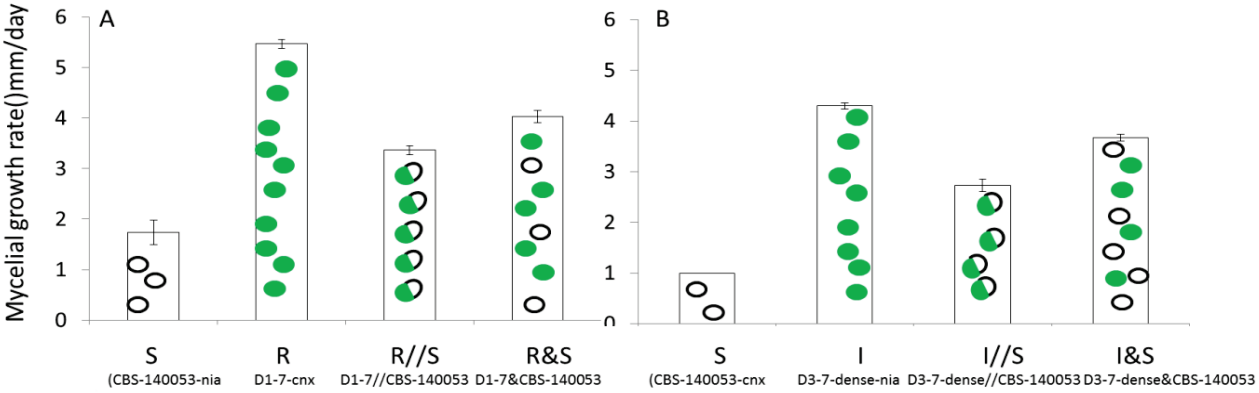


Figure 2. The azole resistance of heterokaryons relative to that of the individual homokaryon and heterozygous diploids was measured as the mycelial growth rate. Strains used were: sensitive(S) CBS-140053, intermediate (I) D3-7#-dense and resistant (R) D1-7# (see Table 1). // : diploid; & : heterokaryon

The nuclear ratio in heterokaryons is flexible

The growth pattern of heterokaryons was strikingly different on medium without azoles compared to that on medium with 1 µg/mL Difeconazole (Figure 3). Whereas erratic growth of the forced heterokaryon was typically seen on medium without azoles, a more compact circular colony was formed on azole-containing medium. The use of a white spore colour mutation in one of the strains in a heterokaryon allowed for direct testing of the nuclear ratio in a heterokaryon on media with or without azoles (Figure 3). The ratio of white to green colonies from spores of heterokaryons S&S (CBS-140053& CBS-140053), I&S (D3-7#-dense& CBS-140053) and R&S (D1-7#&CBS-140053) was analyzed (Figure 4). For the sensitive heterokaryon S&S (CBS-140053& CBS-140053), there was no significant difference between the nuclear ratio on medium with and without 1µg/ml difenoconazole (T- test, $T_{6,2}$ = -2.151 P > 0.05). For the heterokaryons formed between the sensitive and resistant nuclei I&S (D3-7#-dense & CBS-140053) and R&S (D1-7#&CBS-140053), the percentage of intermediate resistant nuclear, I, shifted from 32% to 50%: 1.7 fold (T- test, $T_{6,2}$ = -9.843 P < 0.01) and the percentage of R-nuclei (D1-7#) shifted from 25% to 78%: 3.5 fold (T- test, $T_{6,2}$ = -38.689 P < 0.01). The R (D1-7#) nuclei in R&S (D1-7#&CBS-140053) heterokaryon on medium without azole was significantly lower than S (CBS-140053) as compared to heterokaryon S&S

(CBS-140053 & CBS-140053) (T- test, $T_{6,2}$ = 3.587 P < 0.05), which indicates that the nuclear R (D1-7#) has certain cost of resistance on MM without azole (Figure 4).

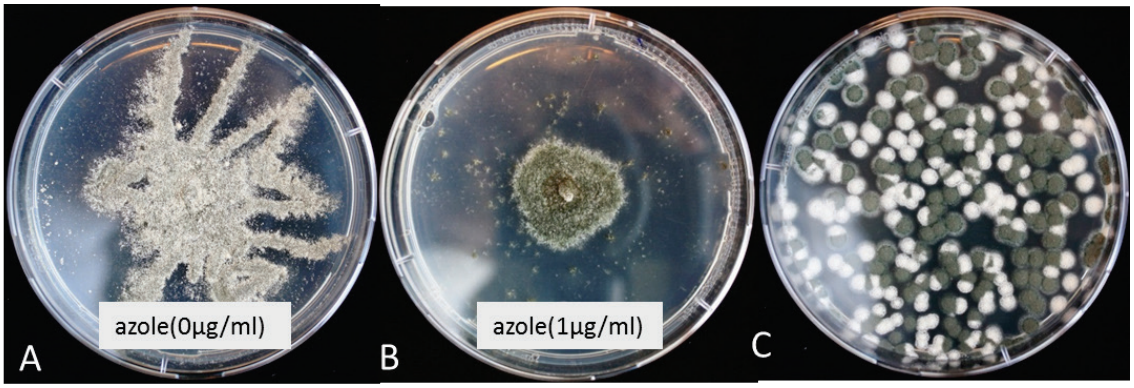


Figure 3. Heterokaryons S- W & R -G (CBS-140053 & D1-7#) growing on selective medium MM without (A) and with (B) difenoconazole (1µg/ml). C: Colonies grown from spores harvested from heterokaryons. W: white-spore head; G: green- spore head. Strains used were: sensitive (S) CBS-140053 and resistant (R) D1-7# (see Table 1).

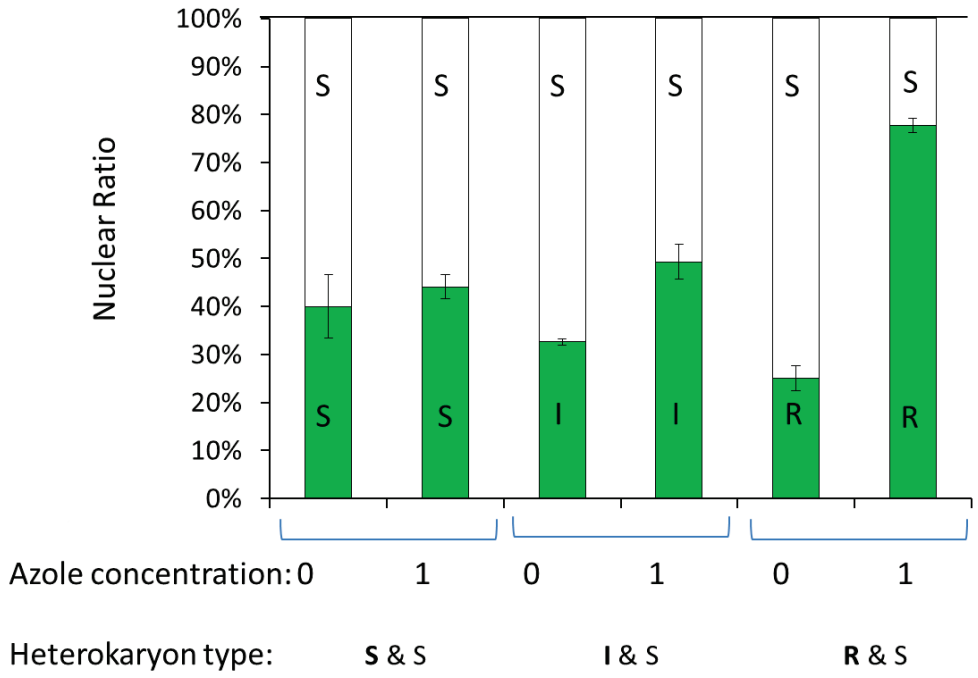


Figure 4. The nuclear ratio changes in three heterokaryons (S & S, I & S and R & S), which were exposure to different concentrations of difenoconazole (0 or 1µg/mL). Strains used were: sensitive (S) CBS-140053, intermediate (I) D3-7#-dense and resistant (R) D1-7# (see Table 1) & : heterokaryon. S&S (CBS-140053& CBS-140053), I&S (D3-7#-dense& CBS-140053) and R&S (D1-7# &CBS-140053).

CHAPTER V

Discussion

In this study, we provide evidence for heterokaryon of *A. fumigatus* both in clinical isolates and under laboratory conditions. Furthermore, we found evidence that heterokaryosis may play a role in adaptation of *A. fumigatus* to an azole-environment: the flexible heterokaryon can adapt to the changing azole environment by altering the nuclear ratio. Which type of nucleus, i.e. azole-resistant or azole-sensitive, is predominant within the heterokaryon is largely determined by the environment the heterokaryon experienced. These observations are relevant for understanding the dynamics of azole resistance in *A. fumigatus* both in the field and in patients.

Polymorphism is regularly found in patient cultures, but the underlying genetic background and possible clinical relevance has long been neglected (Ahmad et al. 2015; Zhang et al. 2015a). Heterokaryons were first described nearly eighty years ago, and are known to be relevant to many different genera in nature (Hansen 1938). Polymorphisms are also observed in evolutionary experiments where *A. fumigatus* cultures are exposed to azoles (Zhang et al. 2015). Here we consider aspects of the heterokaryon for formation, storage and expression of genetic variation in *A. fumigatus* as potential strategy to adapt and persist in a new environment.

A. fumigatus is generally regarded as a generalist saprotrophic fungus that causes, as a side effect of its metabolic versatility, opportunistic infections in immunocompromised patients. Survival of this fungus in a wide range of environments requires an efficient adaptation strategy. Persistence in the human host represents adaptation to the lung environment, and is often accompanied by development of azole resistance in patients receiving chronic azole therapy. In general fungi may adapt to a new environment through creating genetic diversity, through asexual or sexual reproduction, followed by selection of progeny most capable to survive (Kwon-Chung and Sugui 2009; Dyer and O'Gorman 2012). However, sexual reproduction is not relevant to the human environment, and asexual reproduction may occur only when a pulmonary cavity is present. Mostly, the fungus will be present as mycelium and in some clinical conditions, such as in patients with CF, *A. fumigatus* is thought to persist by the formation of biofilms (Muller et al 2010). Microsatellite genotyping indicates that in some patients a single genotype may persist for many years (Mortensen et al 2011). As azole resistance is reported in CF-patients, mutations may have occurred during mitotic divisions in the mycelium giving rise to a heterokaryon. Such heterokaryon can store and further facilitate genetic variation by mutation, parasexual recombination and segregation upon selection pressure. Heterokaryons can formally also arise by anastomosis of hyphae from different cultures, but is restricted by a fungal self/nonself recognition system called heterokaryon incompatibility.

Heterokaryon incompatibility is widespread in fungi and is expressed as the inability to form a stable heterokaryon between different strains of the same species (Glass and

Kuldau 1992; Aanen et al. 2010). As a result of high polymorphism for heterokaryon incompatibility genes in populations, heterokaryon compatibility is mostly restricted to clonally related isolates and, therefore two randomly picked isolates from nature are most likely heterokaryon incompatible (Debets et al. 1994, Van Diepeningen et al. 1997). Indeed, *A. fumigatus* isolates from different patients were heterokaryon incompatible indicating that they were of different clonal origin. However, isolates recovered from the same patient, either in consecutive cultures or different colonies from a single culture could form heterokaryon, despite variability in macroscopic morphology and azole-resistance phenotype. This observation is in line with the observation made by Camps et al. (2012) that nine consecutive isolates from a patient with aspergilloma were isogenic and are therefore expected to be heterokaryon compatible. Also isogenic isolates derived from experimental evolution cultures originating from a common ancestor retained heterokaryon compatibility.

Evolutionary mechanisms underpinning the shift in nucleus ratio under azole selection pressure

Our data show that the proportion of resistant nuclei in heterokaryon rises in response to the azole environment. This has major implications for the understanding of *A. fumigatus* cultures from a patient biopt. It calls into question whether the primary unit of selection is the heterokaryotic mycelium as a whole or each individual nucleus within the mycelium. This may be explained by either direct selection on individual nuclei with fast division, or direct selection of mycelium (sector/ hyphal tips) as a whole.

(I) The selection on individual nuclei

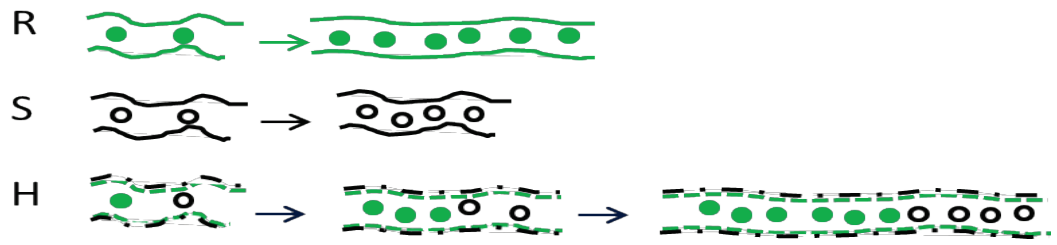
The observation that nuclear ratios can become grossly imbalanced supports the concept that the heterokaryon represents a collective population of nuclei, and the actual "unit of selection" could be each individual nucleus (Pontecorvo 1946; Lewontin 1970; Johannesson and Stenlid 2004). Azoles act by targeting sterol 14α-demethylase CYP51A, which is an important regulatory enzyme in the ergosterol biosynthetic pathway. Hence, the resistant nuclei could produce sufficient ergosterol in the presence of azoles as a result of modification of the CYP51A structure. Division of sensitive nuclei may slow down because of the lack of ergosterol and decrease of the fluidity and permeability of the cell. One explanation is that azole selection triggers the fast division of resistant nuclei in order to sustain the ergosterol production and thus retain hyphal growth. At the next step, due to the difference in nucleus division, after asexual sporulation process, existing ratio shifts in the two types of nuclei may be enhanced and more striking in terms of the resistance phenotype (Figure 5A).

(II) The selection on the sector / hyphal tip

When a heterokaryon originates from an equal mixture of sensitive and resistant nuclei, the sector/ hyphal tip with resistant nuclei may grow faster with sufficient ergosterol supply, as opposed to those harbouring sensitive nuclei which may extend slower. Therefore the hyphal tip with resistant nuclei will grow faster and proceed into the

asexual sporulation process earlier than sensitive hyphal tips. As a result, the sector containing resistant hyphae will produce a larger number of spores, compared with sectors with sensitive hyphae. Overall, when spores are harvested, the proportion of resistant nuclei will outnumber the sensitive nuclei (Figure 5B).

Our data are consistent with previously published observations. Heterokaryons of *N. tetrasperma* possess an additional level of adaptive flexibility to a changing environment, and the phenotype could be altered by changing nuclear ratios(Samils et al. 2014). Early work on ascomycete moulds *Penicillium cyclopium* and *N. crassa* has indicated that nuclear ratios of heterokaryons changed depending on environmental conditions in a manner that reflected the underlying relative fitness of the constituent homokaryons grown in isolation (Jinks 1952; Davis 1960). Hence, carrying both nuclear types and the ability to alter the nuclear ratio enables the fungus to survive in different environments.



Nuclei selection hypothesis

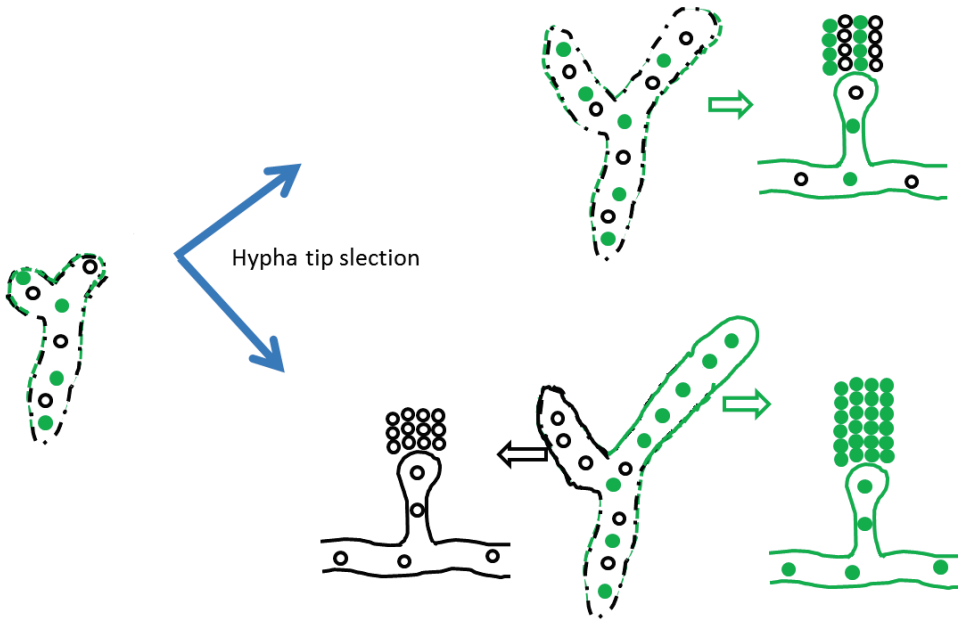


Figure 5. Above: Nuclei selection hypothesis; below: Hyphal tip selection hypothesis.

R (Green nuclei): resistant nuclei; S(white nuclei): sensitive; H: heterokaryon with mixed two types of nuclei. Green cell membrane: healthy cell membrane with good and enough ergosterol. Black cell membrane: unhealthy cell membrane with the decreased fluidity and permeability. In the heterokaryon, the cell membrane is mixed with two types of cell membrane.

Relevance for-azole resistance development

Clinical *A. fumigatus* isolates obtained from clinical specimens were found to be compatible, despite differences in macroscopic morphology. This observation has different consequences. It indicates that azole-resistance development can occur through heterokaryon formation, since new mutations arose in the mycelium. This allows for complementation of the initial cost of resistance and maintenance of genetic variation: depending on the presence of azoles the ratio of sensitive to resistant nuclei will change. But nuclear genotypes will be shielded from strong selection in the heterokaryon, and may persist, be it at low level and contribute to the overall performance of the mycelium. If indeed long-lived mycelium in patients is heterokaryotic, this also has impact on the way fungal infections in patients are analyzed. After plating a biopt sample from a patient a single genotype is not to be expected, and therefore many isolates should be analyzed. Patient-acquired resistance can be proven when a phenotype switch takes place from azole-sensitive to azole-resistant in consecutive isolates recovered from individual patients. This has been observed in isogenic isolates recovered from patients treated with azoles and who have a pulmonary cavity (Camps et al, 2012). It is believed that *de novo* mutations may arise through asexual reproduction, which are subsequently selected in the azole-environment. In culture, commonly different *Cyp51A*-mutations are found, which underscores the efficiency of this adaptation strategy. However, azole-resistance mutations have also been recovered from patients without pulmonary cavities, notably patients with CF (Mortensen et al 2011). *A. fumigatus* is thought to persist in the mucus in the lung through hyphal networks and biofilm (Muller et al 2010). As in CF *A. fumigatus* is probably trapped in the hyphal form, adaptation through heterokaryon formation is a likely alternative strategy.

The flexible heterokaryon may enable *A. fumigatus* to adapt to the lung environment by altering the ratio of each type of nucleus and by complementation between sensitive and resistant nuclei (e.g. of the sensitivity to azoles of the sensitive nuclei and to the cost of resistance of the resistant nuclei, or of different resistance mutations in the different nuclei). Genotyping of *A. fumigatus* strains recovered from CF-patients suggest that variants from a patient are isogenic, and have a heterokaryotic origin. The observation that the resistance level of a mixture of such compatible genotypes differs from each single one indicates heterokaryons may play an important role in the adaptation in *A. fumigatus*. On the other hand, cheating nuclei may also accumulate in a heterokaryon thus attenuating the fitness of the fungal mycelium. Understanding such conditions is very helpful in regulating the resistance unit (spores or hypha) and better managing the azole resistance in *A. fumigatus.*

CHAPTER V

Conclusions and Future Outlook

Our results demonstrate that the full life cycle of *A. fumigatus* needs to be taken into account to understand the adaptation and subsequent persistence of *A. fumigatus* in the human lung. In addition to asexual reproduction, our results indicate that the flexible heterokaryon is another strategy that the fungus can utilize to adapt e.g. to the lung environment and its varying conditions such as azole exposure. Variable macroscopic morphology, which is often found in aspergillus colonies, supports a role of heterokaryosis. Genetic analysis of individual colonies obtained from specimens from CF patients will help us to further understand the role of heterokaryon formation and its relevance for persistence of *A. fumigatus* and (azole) resistance. Understanding the key factors that facilitate resistance selection in *A. fumigatus* is essential to design strategies that prevent or overcome this emerging threat.

Acknowledgements

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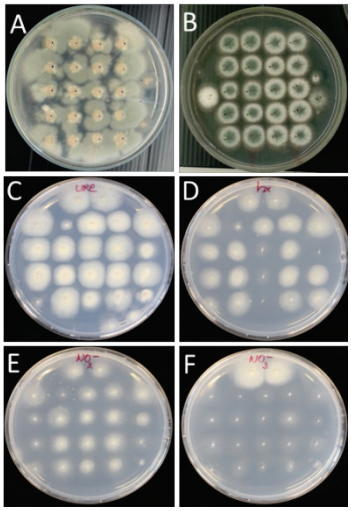
Supplementary data

Supplement 1



A, : Potential Chlorate mutants appear as sector along the main colony after UV radiation 60s. B: Master plate of potential chlorate mutants is ready for Nitrate deficiency test

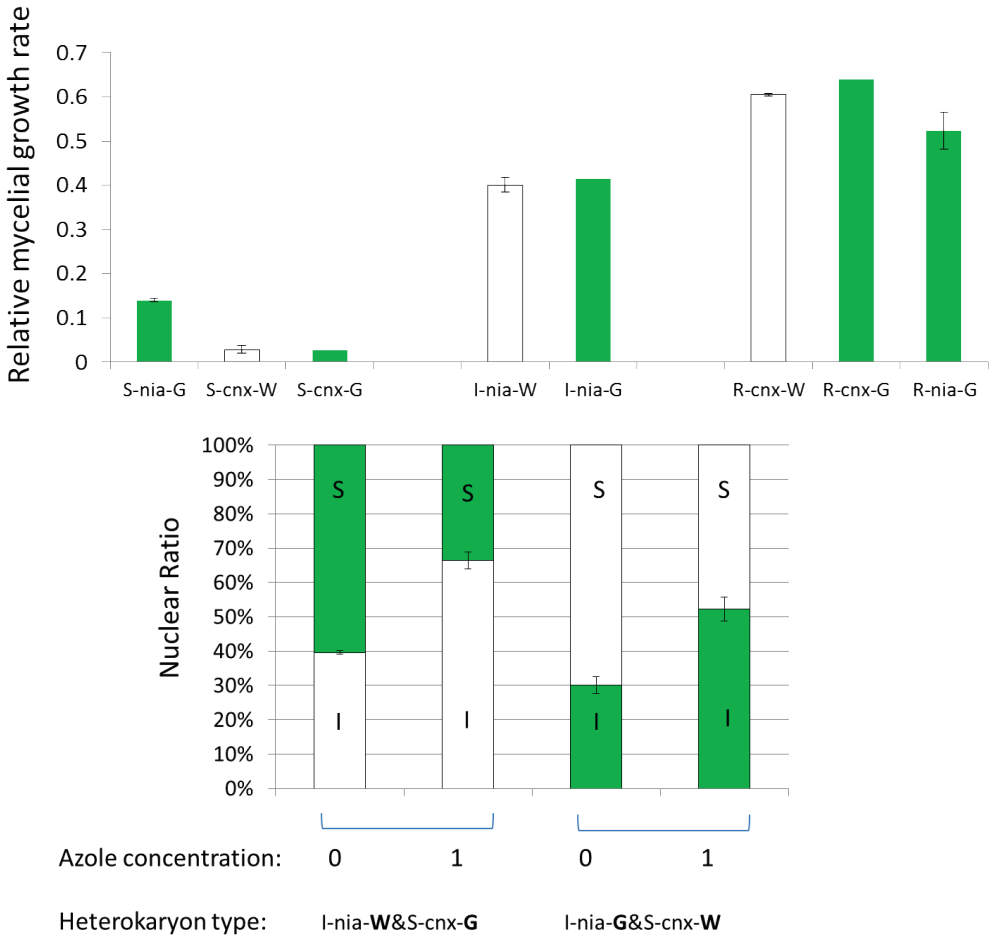
C, D, E, F: Nitrate deficiency test

Supplement 2

The effect of chlorate and color makers on the ratio changes in the heterokaryon

1) The colour influence on the growth rate of mutants and ratio shift in heterokaryon

All chlorate mutants with different colour were grown on MM+ ure ± azole. After 4 days of incubation at 37°C. The mycelial growth rates were recorded. It has shown below, white and green colours do not have influence on the growth rate, (comparison between S-cnx-G and S-cnx-W; I –nia-W and I – nia-G; R-cnx-W and R-cnx-G). In the heterokaryon (I –nia-W&S-cnx-G), in order to check whether colour influence the ratio shift, we exchange the colours of two types of nuclear, hence, the new heterokaryon(I –nia- G & S-cnx-W) was formed. The figure clearly showed the nuclear ratio shift remained the same, when move from no azole environment to azole containing environment, although the ratio slightly differ (40%- 30%) when compare the resistant nia ratio in each heterokaryon alone MM without azole.



Above: The relative mycelial growth rate (MGR) of each mutant with different chlorate marker and colour on the MM +ure +1µg/ml difenoconazole. The relative MGR is defined as the MGR divided by the colony diameter of ancestor, grown on MM +ure medium without azole fungicides.

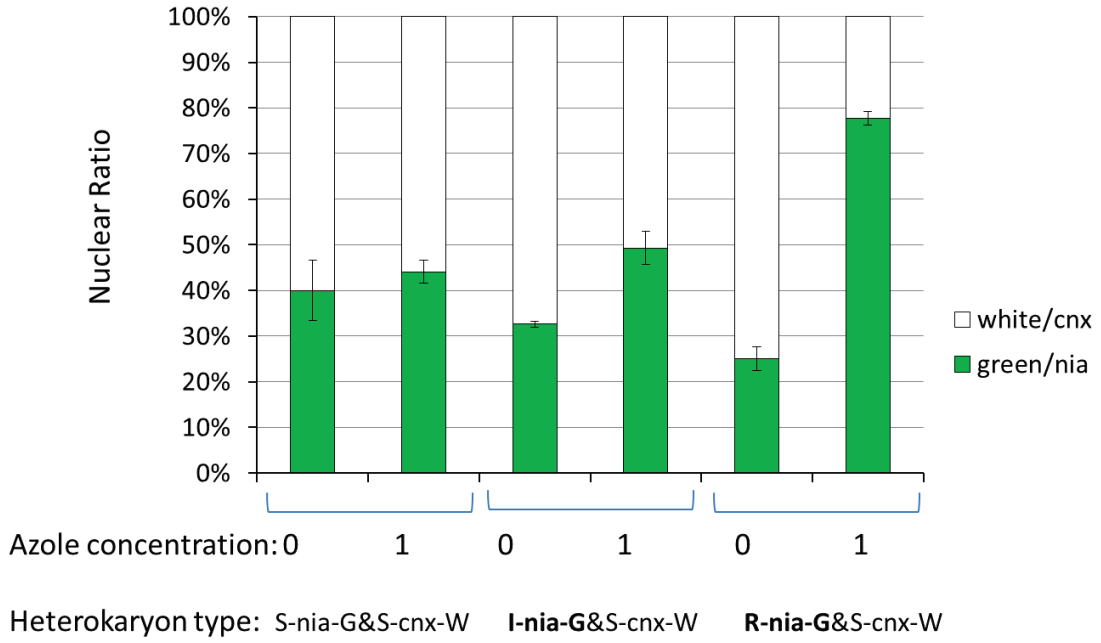
Below: The nuclear ratio shift when colour maker exchange completely in heterokaryons (I –nia-W& S-cnx-G and I- nia-G& S-cnx-W)

2) The chlorate maker influence on the growth rate of mutants and ratio shift in heterokaryon

Two chlorate markers cnx and nia were used in this experiment setup. In above graph it have shown the mycelial growth rate of these two mutants grow alone on MM+ure, in comparison between S-nia-G and S-cnx- G, it showed the growth rate of mutant with cnx maker have significantly higher than mutant with nia, which indicate cnx has negative effect on the growth alone. However, when R-cnx-G and R-nia-G were compared, cnx seems have positive effect.

In sensitive heterokaryon S-nia-G & S-cnx-W, S-nia-G ratio kept around 40% ± 0.07(SEM) when exposed to on MM, interestingly, not expected like 50% to 50%, the ratio of S-cnx –W occupy more than 50%. When this heterokaryon was placed on MM+ 1µg/mL difenoconazole, the S-nia-G ratio shifted slightly, but not significantly. S-cnx –W remains more than 50% percentage. This indicated cnx maker have positive influence on the ratio (figure 4). Maybe cnx also have positive effect in the heterokaryon I-nia-G& S-cnx-

W, R-nia-G& S-cnx-W, but probably it is not obvious when compared with the cost of resistance of I-nia-G and R-nia-G.



The nuclear ratio changes in three heterokaryons (S & S, I & S and R & S), which were exposure to different concentrations of difenoconazole (0 or 1µg/mL). Strains used were: sensitive (S) CBS-140053, intermediate (I) D3-7-dense and resistant (R) D1-7 (see Table 1) & : heterokaryon. S&S (CBS-140053& CBS-140053), I&S (D3-7-dense& CBS-140053) and R&S (D1-7&CBS-140053).

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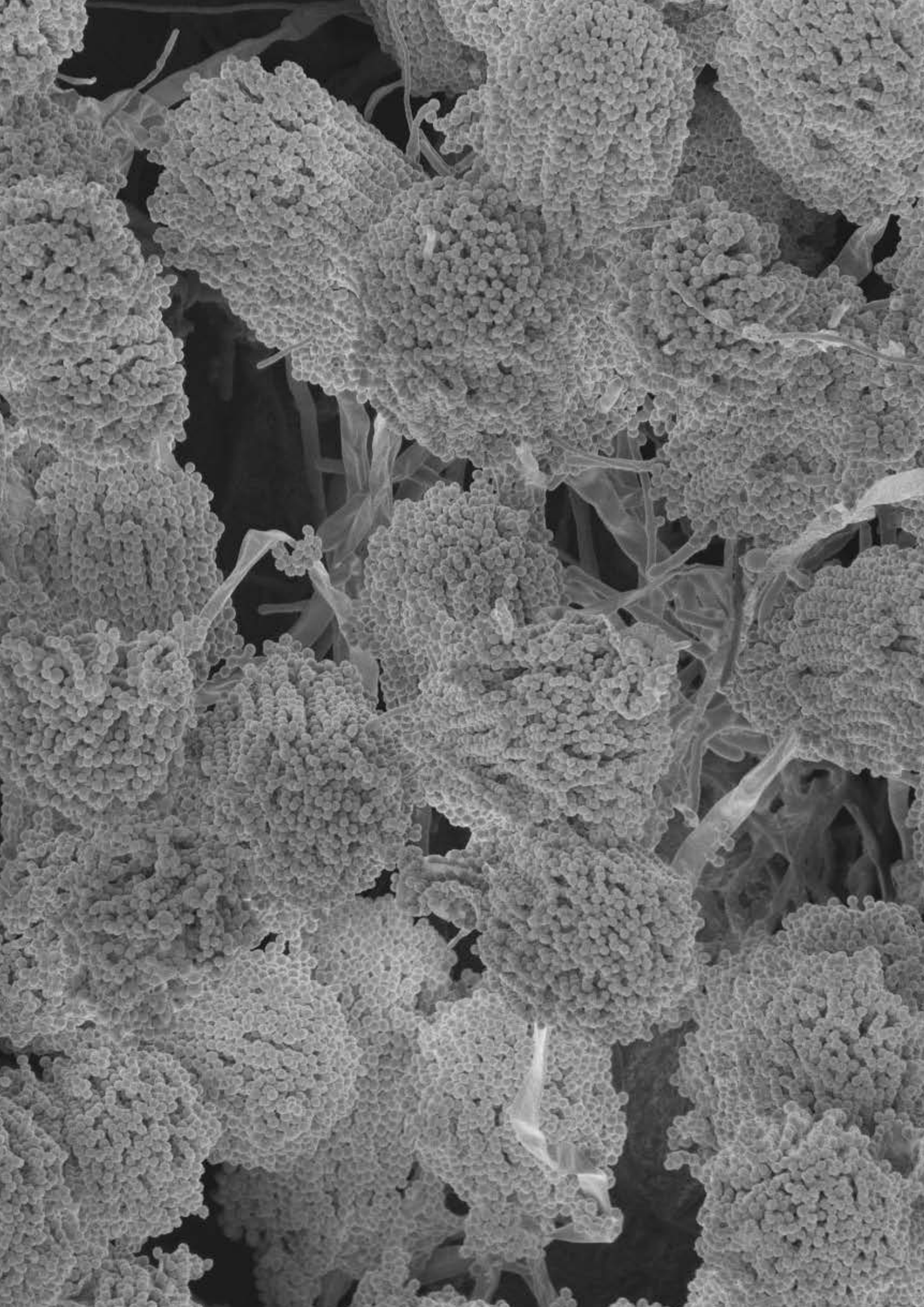

CHAPTER VI

Discovery of a novel environmental azole-resistance mutation in *Aspergillus fumigatus* and the possible role of sexual reproduction in its evolution

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Abstract

We investigated two compost-heaps, one contains azoles and one with organic plant material without azoles, for presence of azole-resistant and azole-sensitive *A. fumigatus* isolates. The azole-free compost yielded 98% (49/50) sensitive and 2% (1/50) azole resistant isolates, whereas the azole-containing compost yielded 8% (4/45) sensitive and 92% (41/45) resistant isolates. From the latter, 84% (37/45) isolates were of the highly resistant TR_{46} Y121F/ T289A genotype and 8% (4/45) had a novel pan–triazole-resistance harbouring a triple tandem repeat: TR_{46}^3/Y121F/ M172I/T289A/G448S. The stark contrast between the two composts indicates that azole–containing compost is a hot spot for the development and maintenance of azole resistance. Subsequent screening of the clinical *A. fumigatus* collection from the Dutch national surveillance programme indicated that this resistance mechanism was already present in 2012, and is now found in all participating medical centres. We were able to recover this novel resistance mechanism with the TR_{46}^3 mutation among the sexual progeny in the lab, from a cross between two isolates of the same compost of opposite mating type both with the TR_{46}. This indicates a role of sex in the emergence of this novel azole–resistance mechanism in *A. fumigatus.* Furthermore, we provide further evidence indicating the potential occurrence of sexual reproduction in compost, but this still needs further confirmation. Our findings alarm the fast development of the azole resistance issue in *A. fumigatus* and further indicate the role of sexual reproduction of *A. fumigatus* in the development of azole resistance.

Key words: *Aspergillus fumigatus*; novel new mutation; compost; azole resistance; hot spot; sexual reproduction; ascospores; conidiospores

Introduction

During the last decade azole resistance has increasingly been reported in *Aspergillus fumigatus* and is now a global public health concern (Snelders et al. 2008; Arendrup et al. 2010; Lockhart et al. 2011; Mortensen et al. 2011; Pfaller et al. 2011; Chowdhary et al. 2012; Morio et al. 2012; Gisi 2013; Bignell 2014; Chowdhary et al. 2014; Wiederhold et al. 2015). Azoles are the cornerstone of treatment of aspergillus diseases and resistance results in increased morbidity and mortality (Howard et al. 2009; van der Linden et al. 2011; van der Linden et al. 2015). Azole resistance may emerge during azole therapy, but the use of azole compounds in the environment is thought to be the major driver of resistance selection in *A. fumigatus* (Snelders et al. 2012; Faria-Ramos et al. 2014). The broad applications of azoles for crop protection, material preservation and possibly hospital use have caused multiple resistance mutations to emerge. The majority of mutations are caused by alterations in the cyp51A gene encoding the target protein sterol 14α-demethylase, such as TR_{34} /L98H and TR_{46} Y121F/ T289A (Diaz-Guerra et al. 2003; Mellado et al. 2004; Chen et al. 2005; Snelders et al. 2008; Snelders et al. 2015; Wiederhold et al. 2015). Figure 1 shows the evolutionary history of promoter and coding region changes in the cyp51A gene of *A. fumigatus* in the Netherlands. These TR variants confer different azole-resistance phenotypes, although most result in a panazole-resistant profile (Vermeulen et al. 2012; Verweij et al. 2012; van der Linden et al. 2013; Verweij et al. 2013; Astvad et al. 2014; Lavergne et al. 2015; Pelaez et al. 2015). The continued emergence of new resistance mutations can only be overcome if the critical steps in resistance selection are understood.

Compost is believed to be an important biological niche for *A. fumigatus* with high densities of conidiospores and azole residues from waste (Millner et al. 1977; Millner et al. 1980). Whether *A. fumigatus* is able to complete the whole life cycle in the harsh and competitive compost environment is unclear. Such knowledge is essential for understanding potential hotspots of development and maintenance of azole resistance in *A. fumigatus*. In addition to azole exposure the mode of reproduction is probably a key factor for resistance selection. We have previously shown that during azole exposure resistance selection is enhanced when *A. fumigatus* reproduces asexually compared with non-sporulating controls (Zhang et al. 2015). Beneficial spontaneous mutations via asexual reproduction were selected and accumulated over time. *A. fumigatus* is an ubiquitous saprophytic mould and has long been regarded as a strictly asexual, 'imperfect', fungus. The last decade there has been accumulating evidence for cryptic sex in this fungus from population genetics studies, genome analysis, and the demonstration of a sexual cycle under laboratory conditions (Varga and Tóth 2003; Paoletti et al. 2005; Kwon-Chung and Sugui 2009; O'Gorman et al. 2009; Dyer and O'Gorman 2011; Dyer and O'Gorman 2012). However, direct observations or sampling of sexual structures in nature have not been reported.

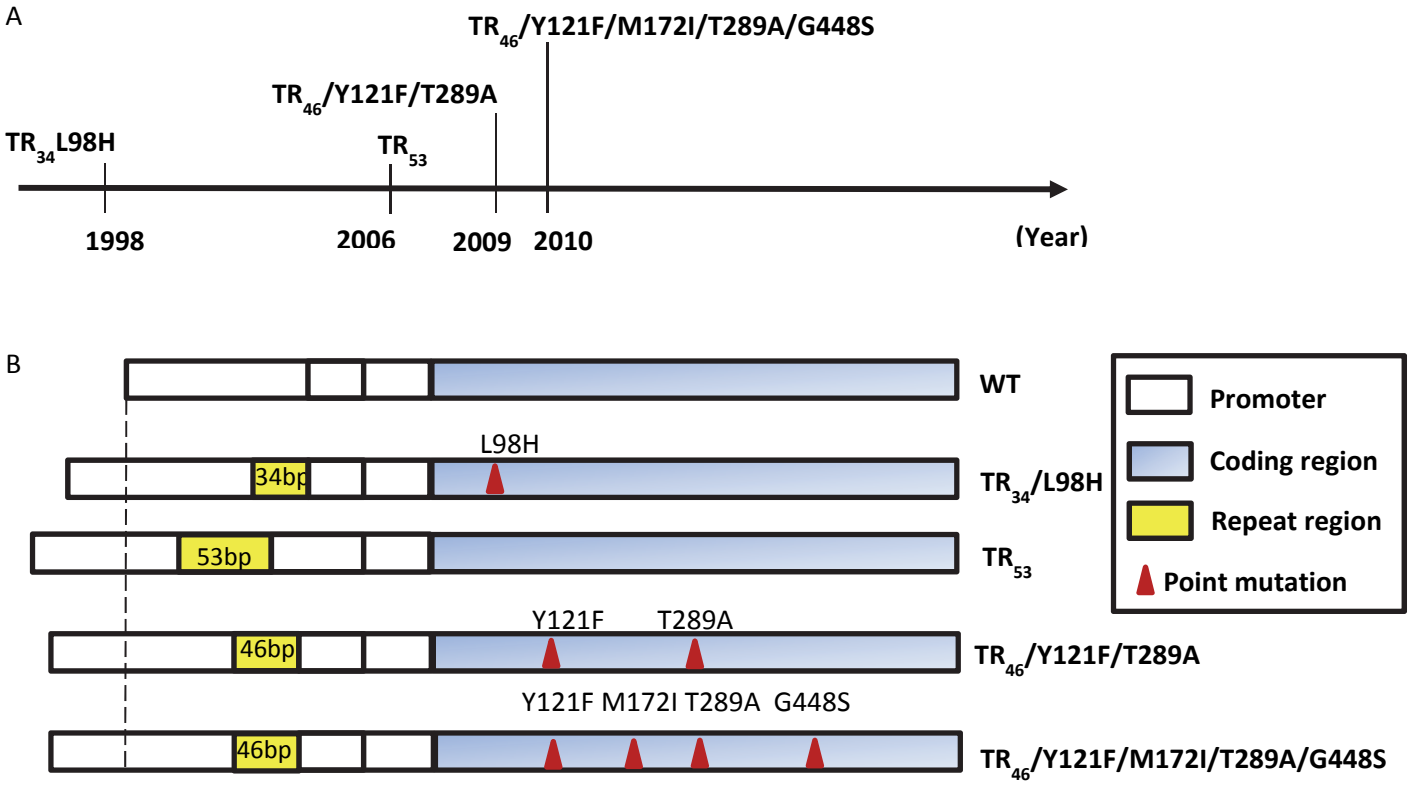


Figure 1. A. The first year of observation of different azole-resistant *A. fumigatus* with TR-promoter mutations in the Netherlands since 1998. B. Genotype illustration of the azole-resistance mutations in the cyp51A gene (promoter and coding region).

In fungi sexual reproduction can enhance adaptation to changing or new environments (Hoekstra 2007; McDonald et al. 2016) as sexual progenies show larger genetic variation compared with asexual populations. Greater genetic diversity is achieved through meiosis, by crossing over and chromosome segregation. Genotyping studies of *A. fumigatus* indeed show high diversity (Varga and Tóth 2003; Losada et al. 2015; Pena et al. 2015; Teixeira et al. 2015), which may be due to sexual reproduction in nature. In addition, both mating types are found in approximately equal proportion in nature. Therefore, the sexual cycle may play a role in the ability of the fungus to adapt to the azole-environment. Compost may provide the right conditions for sexual reproduction.

In this study, we investigated two compost-heaps, with and without azoles exposure respectively. We made use of heat shock to selectively isolate highly diverse genotypes. The genetic diversity of *A. fumigatus* from two composts was compared. This information is vital to understand the hot spot for emergence of azole resistance in *A. fumigatus* and potential role of reproduction mode in the development of azole resistance.

Materials and methods

Sampling and screening for heat-resistant propagules of *A. fumigatus* in azole- and non-azole containing compost

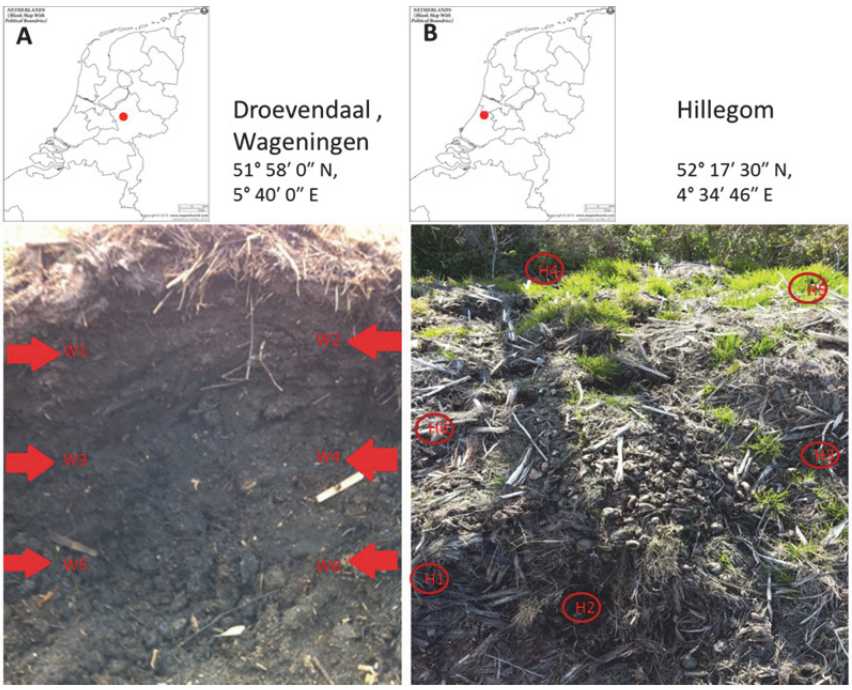


Figure 2. The location and position of samples taken from compost in Wageningen (W1-6) and Hillegom (H1-6)

Six samples were collected from each of two compost heaps, one containing azoles (tulip-bulb waste, Hillegom, The Netherlands) and one free of azoles (Wageningen, the Netherlands, Figure 2, Supplement 1). Samples of 1g compost were screened for *A. fumigatus* before and after heat shock. 10 mL of sterile saline with 0.05% Tween was added into 1g of compost sample. From these suspensions, before and after heat-shock, for 1h at 70^{o}C, 50 µl samples were plated on MEA (malt extract agar: (Zhang et al. 2015)) supplemented with two antibiotics (streptomycin10 µg/ml and tetracycline15 µg/ml, Sigma Aldrich, Germany) and incubated at 37 ^{o}C. This heat shock procedure has been shown to kill *A. fumigatus* conidiospores, but not ascospores if present in the sample (O'Gorman et al. 2009; Kwon-Chung and Sugui 2009). As a consequence, any colony growing after heat shock is likely to have originated from a sexual spore. After two days the colonies were counted and the survival rate was established. The morphology of germinating propagules in heat-shocked compost samples after 9 hours of growth was studied using light microscopy. To test the differential survival of conidiospores and ascospores in compost during heat shock, we performed a control experiment. We added ~10^5 ascospores, from the cross between CBS 140053 and CBS 140054, or

conidiospores, from CBS 140053, to 1 g of autoclaved compost in 10 mL of saline with 0.05% Tween (25 min at 121 °C).

Identification and genotyping of presumed ascospore-derived cultures from compost

We randomly picked 50 cultures, that all showed *Aspergillus* morphology, from a Wageningen (W, azole free) and a Hillegom (H, azole containing) sample respectively. These were identified as *A. fumigatus* by sequencing of the ß-tubulin and carboxypeptidase-5 genes (O'Gorman et al. 2009) as well as the mating-type gene (Paoletti et al. 2005) and by their capacity to grow at 48°C. Next, these were genotyped for six microsatellite markers (STR3 A, B, C and STR4 A, B, C) and the cyp51A gene and its promoter (de Valk et al. 2005: Balajee et al. 2007; Snelders et al. 2009).

Susceptibility testing and cyp51A gene expression of TR variants

Since we previously found that the level of resistance based on mycelial growth rate (MGR) is highly correlated with the results from the MIC test, we here used the more straightforward and reproducible MGR measurement as described before (Zhang 2015). The mycelial growth rate (MGR) of TR variants TR_{34} and TR_{46}, $TR_{46}{}^{3}$, that were isolated from compost heaps, was assayed. Susceptibility of the observed TR variants to triazole drugs was in addition tested in a MIC assay using a broth microdilution method according to EUCAST protocol E.DEF 60 9.1 (Camps et al. 2012a; Zhang et al. 2015). The *cyp51A* expression was analyzed from duplicate cultures of three different strains per TR variant. Expression levels were calculated from Cyp51A/actin mRNA ratios and normalized for WT expression levels (Snelders et al. 2015).

Clinical implications of environmental azole resistance mutations

The presence of new azole resistance mutations that were found in the compost in clinical isolates was investigated using the national aspergillus resistance surveillance. In five Dutch University Medical Centres, clinical *A. fumigatus* isolates are routinely screened for azole-resistant phenotype using agar supplemented with medical triazoles. Isolates that grow in the presence of azoles are sent to Radboud University Medical Centre for MIC-testing and genotypic characterisation. Screening for triazole resistance mutations was performed using the Y121F mutation for TR_{46}/Y121F/T289A and /L98H for the TR_{34}/L98H mutation. These two mutations have been shown to account for over 80% of clinical triazole resistance. If through screening no mutations are found, the cyp51A gene is sequenced. For Y121F positive isolates the accompanying TR was determined using PCR and for selected isolates relevant clinical information was collected.

Genotypic relationship of the resistant *A. fumigatus* isolates TR_{46}^3 Y121F/M172I/T289A/G448S from environmental and clinical samples

Dendrogram: The dendrogram was based on a categorical analysis of six microsatellite markers in combination with unweighted pair group method with arithmetic mean clustering. The scale bar indicates the percentage identity between selected environmental TR_{46}^3 variants isolates vs. clinical isolates (Camps et al. 2012).

Genotypic relationship of *A. fumigatus* TR variants in compost Hillegom

Minimum spanning tree (MST): minimum spanning tree showing the genotypic relationship between all *A. fumigatus* isolates from compost Hillegom (Camps et al. 2012b).

Sexual cross between twoTR_{46} strains of opposite mating type from the same compost

In order to investigate the possibility to generate TR_{46}^3 through sexual reproduction, two pairs of strains from the same compost sample with the TR_{46} resistance mutation and of opposite mating type (isolates H40(MAT1-1) & H35(MAT1-2) and H33(MAT1-2) &H29(MAT1-1)) were used for a sexual cross following the protocol of O'Gorman et al (O'Gorman et al. 2009). After four months, cleistothecia were harvested and 10^5 ascospores were heat shocked, and plated on MEA with posaconazole (2mg/L).

Results

Isolation of *A. fumigatus* from azole-containing and azole-free compost before and after heat shock

Before and after heat shock of each of the six samples, colonies with macroscopic and microscopic characteristics of *A. fumigatus* were recovered. Of each sample, 20 randomly picked colonies were confirmed as *A. fumigatus* based on sequence analysis and their ability to grow at 48°C. Before heat shock each sample contained more than 10^5 CFU. After heat shock, all of the six samples from the non-azole compost heap (W1 to W6) and two of the six samples of the azole compost heap (H1 and H4) yielded *A. fumigatus* isolates ranging from 71 to ~8,000 cfu per 1g of compost (Figure 3).

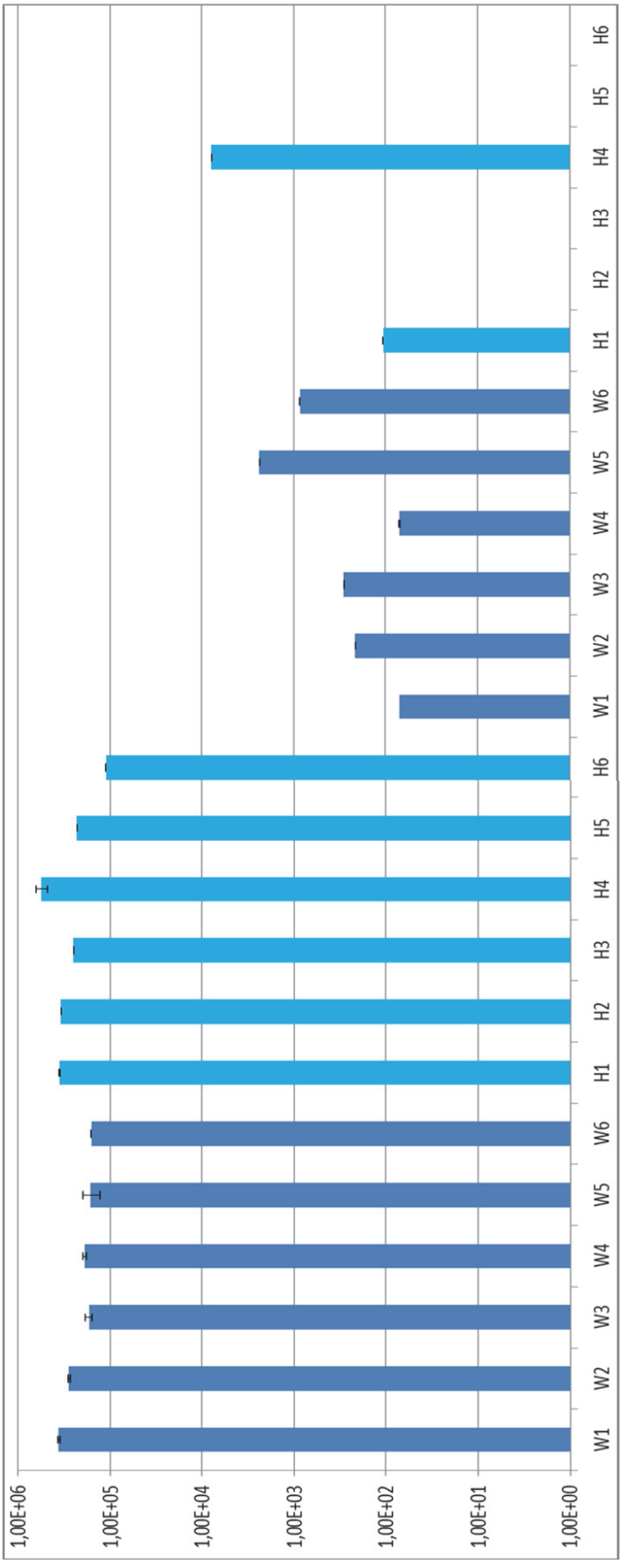
Genotyping of heat-resistant cultures from compost

Azole-resistant *A. fumigatus* was found in both compost heaps, although the proportion of resistance was different, 95% resistance in the azole-exposed compost compared to 2% in the azole-free compost. Genetic characterisation of the azole-resistant isolates revealed the presence of a new mutation, consisting of three copies of TR_{46} (TR_{46}^3) in the promoter and four mutations in the CYP51A: Y121F/M172I/T289A/G448S. The variation in the *cyp51A* gene was higher in the azole-containing compost (8%WT, 84%TR_{46}, 8% TR_{46}^3) than the azole-free compost (98% WT, 2% TR_{34}) (Table 1 and 2). The distribution of MAT1-1: MAT-2 among the different genotypes (11:15 and 15:21 for the azole-containing and azole-free composts respectively) was not significantly different from the expected 1:1 ratio.

Susceptibility testing and gene expression of the TR variants (TR_{34}, TR_{46} and TR_{46}^3)

The mycelial growth rate (MGR) of the TR variantsTR_{34}, TR_{46} and TR_{46}^3 isolated from the two composts is shown in Figure 4. TR_{46}^3 exerted the fast mycelial growth rate on medium supplemented with posaconazole, when compared with TR_{34}/L98H and TR_{46}/Y121F/T289A (ANOVA, P <0.01**, Figure 4). In addition, the high resistance of TR_{46}^3 was confirmed by MIC testing, displaying a pan-triazole-resistant phenotype to posaconazole, itraconazole, and voriconazole(Table 3), even higher than the pan-triazole resistant TR_{34} and TR_{46} (van der Linden et al. 2013).

Expression levels assayed from Cyp51A/actin mRNA ratios showed that even in the absence of azoles, cyp51 A gene expression level in the TR_{46}^3 was significantly higher than WT, TR_{34}/L98H and TR_{46}/Y121F/T289A, which suggests that the repeats in the promoter region are able to generate more CYP51 to cope with azole stress and therefore contributes to a resistant phenotype (Figure 5).



Figur e 3. The density (log of colony-forming units per 1 g of compost) of before and after heat-shock propagules of *A. fumigatus* in samples taken at different positions from azole-free (W1-W6) and azole-containing (H1-H6) compost

Table 1 The genetic variation in azole-containing compost from flower-bulb waste in Hillegom, The Netherlands.

AZN_NMR	sample number	STR3			STR4			Cyp51A promoter	Cyp51A SNP PCR	Mating type	Genotype
		A	B	C	A	B	C				
V181-07	H01	33	10	23	6	13	8	TR_{46}	Y121F	MAT1-2	1
V181-08	H02	44	8	61	6	12	8	TR_{46}	Y121F	MAT1-2	2
V181-09	H03	44	8	7	10	9	7	TR_{46}	Y121F	MAT1-2	3
V181-10	H04	44	8	7	10	9	7	TR_{46}	Y121F	MAT1-2	3
V181-11	H05	42	8	61	7	10	8	WT	NONE	MAT1-1	4
V181-12	H06	35	9	27	8	10	8	WT	NONE	MAT1-1	5
V181-13	H07	44	8	12	10	8	18	TR_{46}	Y121F	MAT1-2	6
V181-14	H08	35	11	16	8	7	6	WT	NONE	MAT1-1	7
V181-15	H09	42	9	62	10	8	7	TR_{46}	Y121F	MAT1-2	8
V181-16	H10	46	8	7	10	8	7	TR_{46}	Y121F	MAT1-1	9
V181-17	H11	45	8	7	10	8	7	TR_{46}	Y121F	MAT1-2	3
V181-18	H12	44	8	7	10	8	7	TR_{46}	Y121F	MAT1-2	3
V181-19	H13	43	8	11	10	8	18	TR_{46}	Y121F	MAT1-2	10
V181-20	H14	33	10	23	6	10	13	TR_{46}	Y121F	MAT1-2	11
V181-21	H15	44	8	7	10	9	7	TR_{46}	Y121F	MAT1-2	3
V181-22	H16	42	8	61	10	7	7	TR_{46}	Y121F	MAT1-2	8
V181-23	H17	31	8	21	6	12	8	TR_{46}	Y121F	MAT1-2	12
V181-24	H18	44	8	7	10	8	7	TR_{46}	Y121F	MAT1-2	3
V181-25	H19	44	8	61	8	10	8	WT	NONE	MAT1-1	4
V181-26	H20	42	8	61	10	9	7	TR_{46}	Y121F	MAT1-1	9
V181-27	H21	44	9	7	10	7	7	TR_{46}	Y121F	MAT1-2	3
V181-28	H22	44	8	7	10	7	7	TR_{46}	Y121F	MAT1-2	3
V181-29	H23	44	8	7	10	9	7	TR_{46}	Y121F	MAT1-2	3
V181-30	H24	26	8	12	6	7	7	**TR_{46}^{3}**	Y121F	MAT1-1	14
V181-31	H25	33	10	23	6	13	9	TR_{46}	Y121F	MAT1-2	1
V181-32	H26	44	8	12	2	8	6	TR_{46}	Y121F	MAT1-2	16
V181-33	H27	46	8	12	2	8	8	TR_{46}	Y121F	MAT1-2	16
V181-34	H28	55	8	7	6	8	8	TR_{46}	Y121F	MAT1-1	17
V181-35	H29	44	8	7	12	9	9	TR_{46}	Y121F	MAT1-2	18
V181-36	H30	26	8	12	6	9	7	**TR_{46}^{3}**	Y121F	MAT1-1	14
V181-37	H31	44	8	7	10	8	7	TR_{46}	Y121F	MAT1-2	3
V181-38	H32	26	7	12	6	8	7	TR_{46}	Y121F	MAT1-1	19
V181-39	H33	41	8	7	10	8	8	**TR_{46}^{3}**	Y121F	MAT1-2	20
V181-40	H34	25	18	24	8	10	9	TR_{46}	Y121	MAT1-1	21
V181-41	H35	31	8	21	6	13	9	TR_{46}	Y121F	MAT1-2	12
V181-42	H36	44	8	7	10	8	7	TR_{46}	Y121F	MAT1-2	3
V181-43	H37	22	4	13	6	9	9	**TR_{46}^{3}**	Y121F	MAT1-1	22
V181-44	H38	31	8	23	6	13	8	TR_{46}	Y121F	MAT1-2	23
V181-45	H39	44	8	7	10	8	7	TR_{46}	Y121F	MAT1-2	3
V181-46	H40	26	8	21	11	8	7	TR_{46}	Y121F	MAT1-1	24
V181-47	H41	33	10	23	6	13	8	TR_{46}	Y121F	MAT1-2	1
V181-48	H42	44	8	7	9	9	9	TR_{46}	Y121F	MAT1-2	25
V181-49	H43	24	6	20	12	8	7	TR_{46}	Y121F	MAT1-1	26
V181-50	H44	44	8	12	10	8	7	TR_{46}	Y121F	MAT1-2	15
V181-51	H45	44	8	17	2	3	7	TR_{46}	Y121F	MAT1-2	13

Table 2 The genetic variation in azole-free compost from an organic plant waste compost heap in Wageningen, The Netherlands

AZN_NMR	Sample number	STR3			STR4			Cyp51A promot	Cyp51A SNP PCR	Mating type	Genotype
		A	B	C	A	B	C				
V182-18	W1	34	9	8	7	8	31	WT	WT	MAT1-2	1
V182-19	W2	38	8	12	8	10	20	WT	WT	MAT1-1	2
V182-20	W3	30	8	11	8	9	20	WT	WT	MAT1-2	3
V182-21	W4	29	8	12	7	9	20	WT	WT	MAT1-2	3
V182-22	W5	38	10	46	10	8	8	WT	WT	MAT1-1	4
V182-23	W6	29	7	11	9	9	18	WT	WT	MAT1-1	5
V182-24	W7	26	10	21	8	7	5	WT	WT	MAT1-2	6
V182-25	W8	30	8	11	8	9	20	WT	WT	MAT1-2	7
V182-26	W9	35	8	8	7	9	12	WT	WT	MAT1-1	3
V182-27	W10	25	8	8	6	9	8	WT	WT	MAT1-2	8
V182-28	W11	13	18	8	7	26	5	WT	WT	MAT1-2	9
V182-29	W12	30	8	11	5	9	20	WT	WT	MAT1-2	10
V182-30	W13	9	8	8	8	8	6	WT	WT	MAT1-2	11
V182-31	W14	30	8	8	7	9	19	WT	WT	MAT1-1	12
V182-32	W15	13	8	10	8	8	3	WT	WT	MAT1-1	13
V182-33	W16	30	8	8	5	9	19	WT	WT	MAT1-1	14
V182-34	W17	27	8	30	7	6	5	WT	WT	MAT1-2	15
V182-35	W18	27	8	26	10	8	5	WT	WT	MAT1-2	16
V182-36	W19	24	8	8	8	11	33	WT	WT	MAT1-1	17
V182-37	W20	50	8	8	8	9	25	WT	WT	MAT1-2	18
V182-38	W21	10	8	8	5	7	18	WT	WT	MAT1-1	19
V182-39	W22	30	8	11	5	7	18	WT	WT	MAT1-2	20
V182-40	W23	36	10	30	22	9	8	WT	WT	MAT1-1	21
V182-41	W24	30	18	8	10	26	5	WT	WT	MAT1-2	12
V182-42	W25	50	8	8	10	8	5	WT	WT	MAT1-1	23
V182-43	W26	8	7	11	7	8	10	WT	WT	MAT1-1	24
V182-44	W27	26	8	8	8	9	33	WT	WT	MAT1-1	25
V182-45	W28	30	8	11	8	9	21	WT	WT	MAT1-2	26
V182-46	W29	25	8	21	14	8	5	WT	WT	MAT1-2	19
V182-47	W30	48	12	8	10	26	20	WT	WT	MAT1-1	3
V182-48	W31	27	8	26	10	8	5	WT	WT	MAT1-2	28
V182-49	W32	27	8	26	10	8	5	WT	WT	MAT1-2	29
V182-50	W33	27	8	26	9	8	5	WT	WT	MAT1-2	18
V182-51	W34	28	10	8	8	8	8	WT	WT	MAT1-1	18
V182-52	W35	29	16	8	10	26	5	WT	WT	MAT1-2	18
V182-53	W36	30	8	11	8	9	21	WT	WT	MAT1-2	30
V182-54	W37	38	8	12	5	10	21	WT	WT	MAT1-1	24
V182-55	W38	32	8	8	7	9	19	WT	WT	MAT1-2	3
V182-56	W39	32	8	8	8	9	19	WT	WT	MAT1-2	31
V182-57	W40	28	9	12	8	6	11	TR_{34}	L98H	MAT1-1	14
V182-58	W41	36	8	9	7	9	8	WT	WT	MAT1-2	14
V182-59	W42	45	9	10	10	8	10	WT	WT	MAT1-1	33
V182-60	W43	37	8	20	6	7	5	WT	WT	MAT1-1	34
V182-61	W44	29	8	11	8	9	27	WT	WT	MAT1-2	35
V182-62	W45	31	8	7	6	8	18	WT	WT	MAT1-1	36
V182-63	W46	34	8	8	5	9	8	WT	WT	MAT1-2	37
V182-64	W47	37	22	21	6	8	5	WT	WT	MAT1-2	38
V182-65	W48	39	8	8	8	9	21	WT	WT	MAT1-2	27
V182-66	W49	15	8	20	5	7	5	WT	WT	MAT1-2	22
V182-67	W50	26	10	8	31	29	6	WT	WT	MAT1-2	32

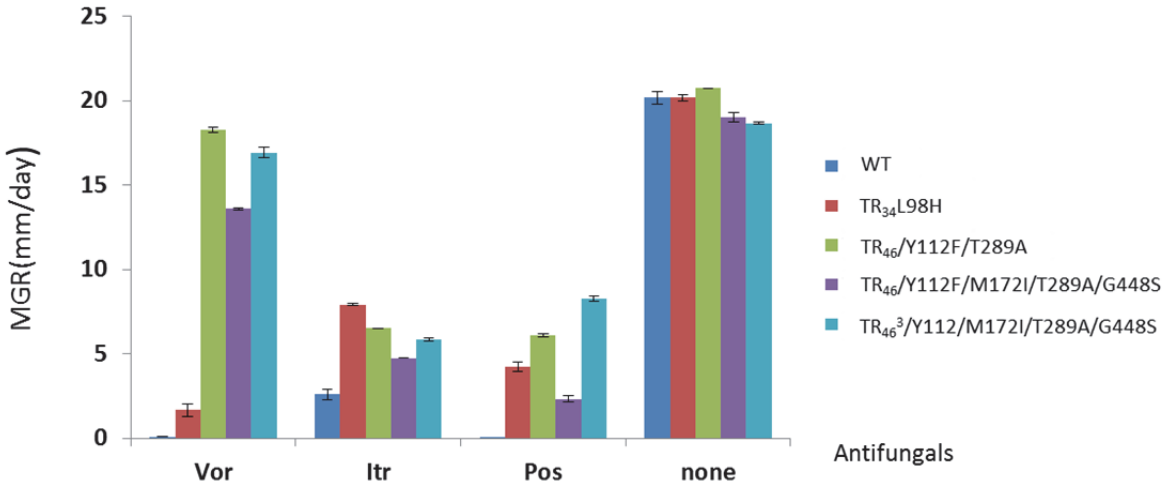


Figure 4. The MGR assay of three TR variants: TR_{34} and TR_{46}, TR_{46}^3 against voriconazole, itraconazole, and posaconazole. (MEA supplemented with 1mg/L respectively)(ITR, itraconazole; VOR, voriconazole; POS, posaconazole; none: without any azoles)

Table 3. The in vitro activity of itraconazole, voriconazole, and posaconazole against three TR variants: TR_{34}, TR_{46}, and TR_{46}^3.

MIC range (mg/l)	ITR	VOR	POS	
TR_{34}/L98H	>16	4-8	0.25-0.5	Snelders et al.2009
TR_{46}/Y121F/T289A	4/16	>16	0.25-2	van der Linden et al. 2013
TR_{46}^3/Y121F/M172I/T289A/G448S	> 16	>16	1	This study compost+ clinical

(ITR, itraconazole; VOR, voriconazole; POS, posaconazole)

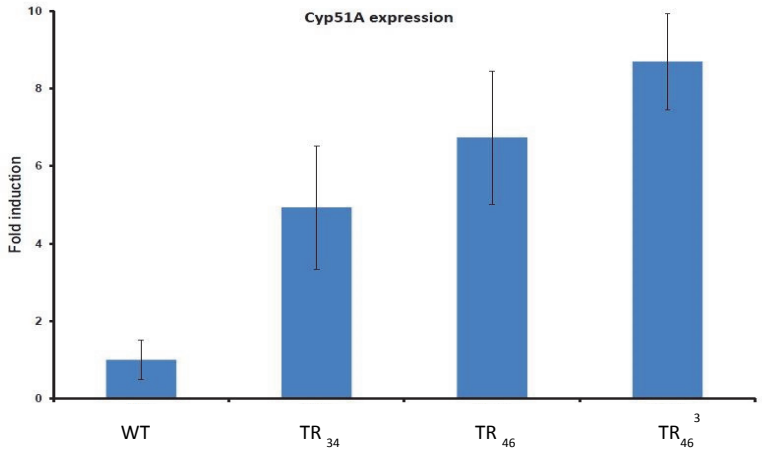


Figure 5. The overexpression of cyp51A in TR variants (WT TR_{34}, TR_{46}, and TR_{46}^3)

Clinical implications: analysis of clinical isolates from the Dutch national surveillance program and discovery of TR_{46}^3 Y121F/M172I /T289A/G448S in two patient isolates

The *A. fumigatus* culture collection of the Dutch national resistance surveillance was used to determine if TR_{46}^3 Y121F/M172I/T289A/G448S was present in clinical isolates. All clinical *A. fumigatus* isolates collected between 2013 and 2015 that harboured a Y121F-mutation were investigated for the presence of TR_{46}^3 by PCR. Among 98 Y121F-harbouring isolates, three we identified with TR_{46}^3. In addition one isolate was found with four TRs (TR_{46}^4) in combination with the four mutations in the cyp51A-gene, TR_{46}^4/ Y121F/M172I/T289A/G448S.

One TR_{46}^3/ Y121F/M172I/T289A/G448S isolate was recovered from a sputum sample from a 34-year-old female in 2013 in the western part of the Netherlands. The patient was hospitalized for treatment of synovial cell sarcoma with pleural metastasis. Computed tomography of the lungs showed no evidence for *Aspergillus* disease. A second patient with an *A. fumigatus* isolate harbouring TR_{46}^3/ Y121F/M172I/T289A/G448S was found in 2014 in another hospital in the north eastern part of the country. The isolate was cultured from the right ear of a 34-year-old male who was treated for cholesteatoma, without evidence for *Aspergillus* disease. A third patient was admitted to a hospital in the north west of the country in 2015. The patient was treated for asthma as out-patient because of shortness-of-breath. CT of the chest showed no evidence for *Aspergillus* disease.

A fourth patient was identified through consultation of the mycology expert centre. An *A. fumigatus* isolate was sent in for MIC-testing and was found to harbour the TR_{46}^3/ Y121F/M172I/T289A/G448S mutation. The isolate was cultured from a 43-years-old patient with COPD admitted to a hospital in the east of the Netherlands. The isolate was cultured in 2012. The patient was admitted with a community-acquired pneumonia, and received therapy with posaconazole in addition to antibacterial therapy. Because sputum culture remained positive after two weeks of posaconazole therapy, the strain was sent for MIC-testing. However, a definite diagnosis of invasive pulmonary aspergillosis could not be made.

The TR_{46}^4 isolate was cultured from the ear from a patient with chronic otitis and a open cavity. There fungus may have contributed to the chronic infection, but there was no evidence for invasive disease.

The relatedness between the environmental and clinical TR_{46}^3 / Y121F/M172I/T289A/G448S isolates

The relatedness of all TR_{46}^3 isolates was tested and data are shown in Figure 6. Microsatellite typing showed that almost all environmental TR_{46}^3 clustered together except one isolate (V181-39) showed lower variations in the microsatellite locus. However, the four clinical strains exhibited larger variations in terms of microsatellite

locus. The relatedness factor ranged from 33.3 to 66.7. The environmental TR_{46}^{3} (V181-30, 36 and wg-9, 10, 24, 30, 32, 37) and clinical TR_{46}^{3} (CWZV182-69) strain exhibited a similar fingerprint pattern in the microsatellite analysis (Figure 6).

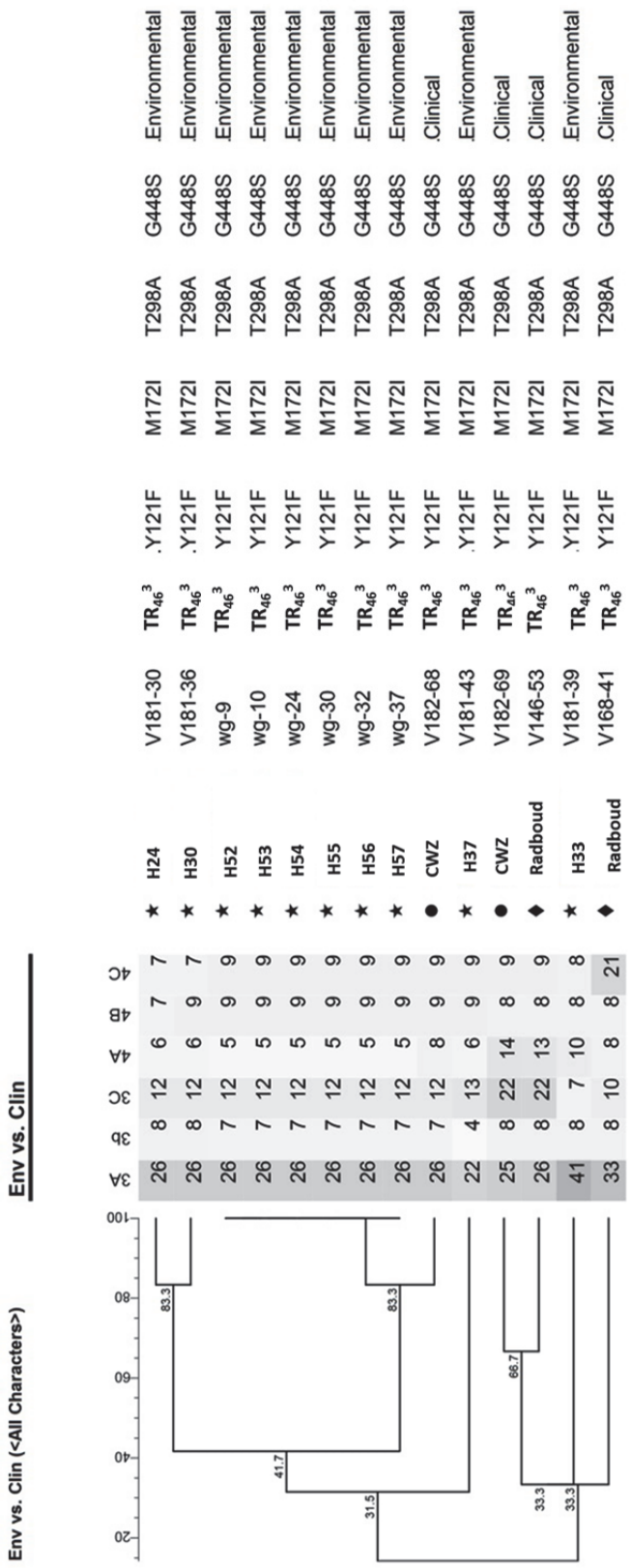


Figure 6. Genotypic relationship between clinical (from CWZ and Radboud Medical Center) and environmental TR_{46}^{3} / Y121F/M172I/T289A/G448S *A. fumigatus* isolates (from azole-containing compost in Hillegom, see Table 1) based on microsatellite genotyping

Genotypic relatedness of TR variants (WT, TR_{46}, and $TR_{46}{}^{3}$) in compost Hillegom

A minimum spanning tree (MST) was constructed based on the six STRAf microsatellite loci of all TR variants (WT, TR_{46}, and $TR_{46}{}^{3}$) in compost Hillegom (45 isolates). The part of TR_{46} (V181-08, 23, 44) clustered with WT (V181-25, 11), which indicates that TR_{46} might origin from the WT. Similarly, the $TR_{46}{}^{3}$ (V181-30, 39) also clustered with TR_{46} (V181-38, 34), which suggests the possible origin of $TR_{46}{}^{3}$ from TR_{46} (Figure 7).

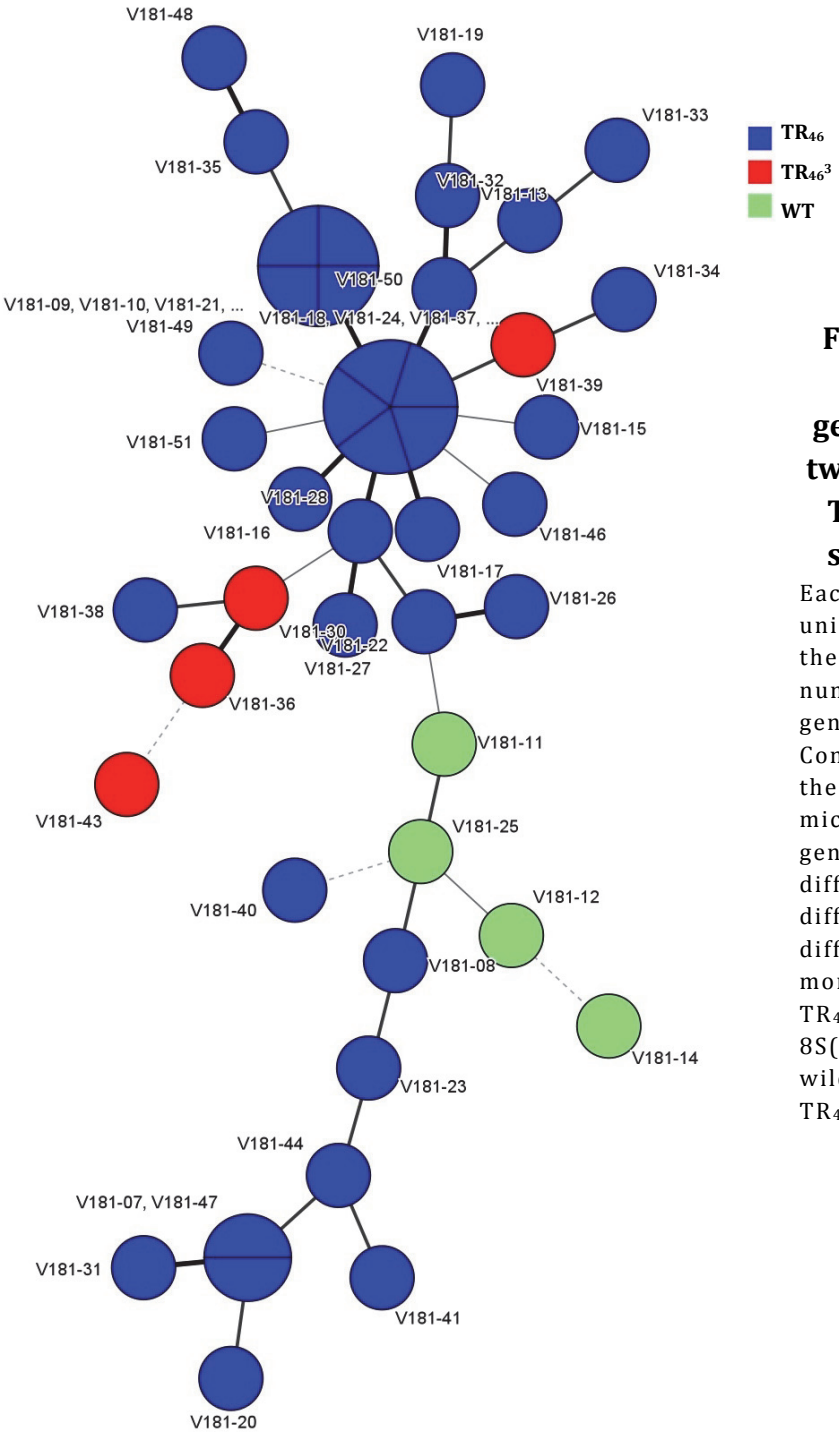


Figure 7. Minimum spanning tree showing the genotypic relationship between the *A. fumigatus* WT, TR_{46}, and $TR_{46}{}^{3}$ from the same compost Hillegom.

Each circle corresponds to a unique genotype, and the size of the circle corresponds to the number of isolates with that genotype (1, 2, or 3 isolates). Connecting lines correspond to the number of different microsatellite loci between the genotypes. Short bold line, 1 difference; black line, 2 differences; long gray line, 3 differences; dotted line, 4 or more differences. Red, $TR_{46}{}^{3}$/Y121F/M172I/T289A/G448S(n=4); green, azole-susceptible wild type (n=4); blue, TR_{46}/Y121F/ T289A (n = 37).

Is sexual reproduction in compost the possible origin of TR_{46}^3 /Y121F/M172I/T289A/G448S?

Evidence that the heat-shock resistant propagules in compost are sexual ascospores

(1) Ascospore-derived colonies are expected to show high genotypic variation.

A high genetic diversity was found in both compost heaps as shown in Table 1 and 2 in terms of *cyp51A* gene and promoter region, microsatellite locus and mating type genes. Within this population a high diversity and equal mating-type distribution is consistent with sexual, rather than asexual reproduction. In addition, the discovery of isolates of opposite mating type with TR_{46}^3/Y121F/M172I/T289A/G448S suggests a potential origin by sex.

(2) TR_{46}^3 can be obtained from a sexual cross between two TR_{46} strains of opposite mating type from the same compost sample

After four months of incubation, following a sexual cross between a pair of TR_{46} strains derived from the azole-containing compost-heap, ~10^5 sexual progenies were plated on MEA supplemented with posaconazole (2 mg/L). Colonies that grew in the presence of posaconazole were selected for sequence analysis of the *cyp51A*-gene. One of which had the novel TR_{46}^3 mutation. This new resistance genotype may have resulted from unequal cross over (Figure 8).

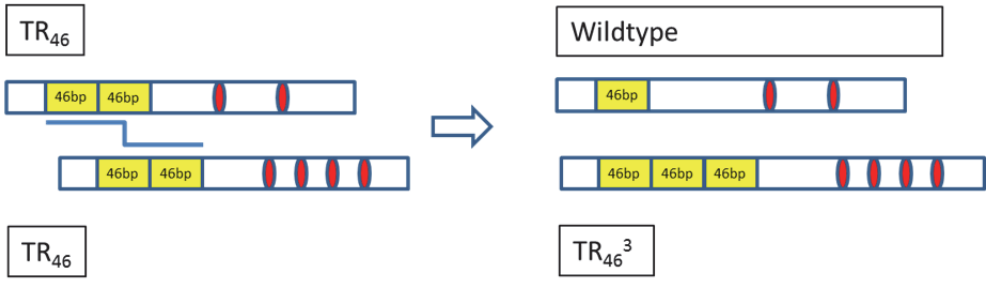


Figure 8. Scenario indicating how unequal crossing over in a sexual cross between two strains with a double repeat TR_{46} can result in a rare meiotic recombinant with the triple repeat TR_{46}^3.

Whether the heat-shock resistant propagules in compost are sexual ascospores remains an open question

(1) It is believed that ascospores, but not conidiospores are resistant to a heat shock of 1h at 70°C (O'Gorman et al. 2009; Sugui et al. 2011). However, from the reconstruction experiment we found that though indeed the ascospores are heat resistant, not all of the 10^5 conidiospores in compost or in saline were killed after 1h at 70°C. A previous study has also shown that a population of conidiospores may not completely killed by heat shock, depending on many factors, like the freshness and concentration of

conidiospores, , the type of strain and the pH (Tsukahara 1975). Therefore, we cannot conclude that all colonies derived after heat shock of compost material have originated from ascospores.

(2) The morphological difference between germinating ascospores and conidiospores was distinguished via two crests (ridges). However, in light microscopy preparations of compost suspensions, structures reminiscent of ascospores were seen (Figure 9, green arrow), but the compost material interfered too much to convincingly discriminate between conidia and ascospores.

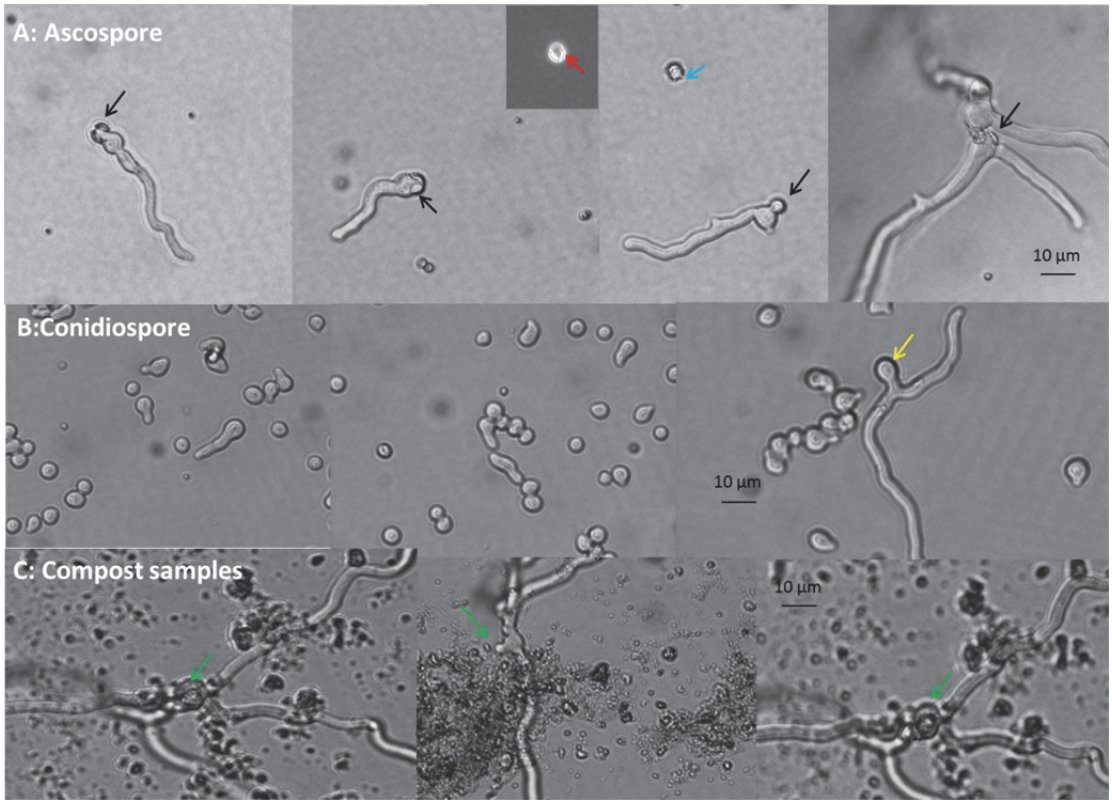


Figure 9. Crests differentiate germinating ascospores from conidiospores.

A: Ascospore; B: Conidiospore; C: Compost sample

Discussion

In this study, we observed that compost with known azole exposure harbours predominantly azole-resistant *A. fumigatus*, whereas in azole-free compost almost exclusively wild type *A. fumigatus* is found. Our study suggests that azole exposure to *A. fumigatus* in its natural habitat at least sustains the presence of azole resistance. This and similar habitats could act as 'hot spot', i.e. provide a source of azole-resistant *A. fumigatus* from which air-borne conidia may migrate and cause disease in susceptible hosts.

We were able to identify a new resistance mutation in the azole-exposed compost. Analysis of clinical isolates that had been collected in the national surveillance program showed that since 2012 each year one patient harboured an isolate harbouring this new resistance mechanism. Although the migration of the $TR_{46}{}^{3}$/Y121F/M172I/T289A/G448S was not rapid, the isolate was recovered from unrelated patients in three different hospitals in the Netherlands. This indicates that the resistance mutation has migrated across the country. Little is known about the migration rate of resistance traits in *A. fumigatus*. The TR_{34}/L98H is the most frequently reported mutation, and is now found globally (Snelders et al. 2008; Arendrup et al. 2010; Lockhart et al. 2011; Mortensen et al. 2011; Pfaller et al. 2011; Chowdhary et al. 2012; Morio et al. 2012; Gisi 2013; Bignell 2014; Chowdhary et al. 2014; Wiederhold et al. 2015). The TR_{46}/Y121F/T289A mutation also showed rapid migration as after its discovery in December 2009, the mutation was recovered from patients from six different hospitals in the Netherlands within one year (van der Linden et al. 2013). Although we found only patient colonization, we believe that isolates harbouring $TR_{46}{}^{3}$ /Y121F/M172I/T289A/G448S will ultimately cause invasive infections as the resistance trait has probably survived in the environment for several years, in competition with other azole-resistant or wild type isolates, indicating comparable fitness.

Analysis of the collection of azole-resistant clinical isolates revealed another new mutation with four copies of the TR_{46}. Although the $TR_{46}{}^{4}$ mutation has not yet been found in the environment, we believe that this mutation also originated through azole exposure to *A. fumigatus* in the environment. These observations indicate that our current azole fungicide use is not durable and that new resistance mutations will continue to emerge. The clinical consequences are that the role of medical azoles in the management of *Aspergillus* diseases will be increasingly threatened. As the arsenal of available drugs to treat *Aspergillus* diseases is already very limited, excess mortality due to azole resistance can be expected (Verweij etal. 2015). Indeed, case series show high mortality rates in patients with azole-resistant invasive aspergillosis (Jan W.M. van der Linden etal. 2011). Environmental resistance is especially difficult to manage as it is found in any *Aspergillus* disease and may occur in any patient, even those without previous azole therapy (Verweij etal. 2016). Recently mixed infections due to azole-susceptible and azole-resistant *A. fumigatus* were reported which further complicates timely diagnosis and successful therapy (Kolwijck et al. 2016).

In order to tackle the problem of emerging azole resistance, it is important to identify hot spots in the environment and to understand how resistance mutations arise and which factors facilitate the geographical migration of mutations.

Possible origins of $TR_{46}{}^{3}$ /Y121F/M172I/T289A/G448S

With regard to the origin of resistant mutants TR_{34}/L98H, TR_{46}/Y121F/T289A and $TR_{46}{}^{3}$ /Y121F/M172I/T289A/G448S, with the combination of TR and point mutation(s), TR could be the consequence of unequal crossing over during sexual or parasexual

recombination. The duplications in the promoter region resulting in the increased expression of the *cyp51* gene may compensate for the reduced CYP51 enzyme activity due to the amino acid changes that reduced the azole sensitivity. Therefore, the TR mutations in the promoter may be seen as compensatory mutations to the azole-resistance point mutations in *cyp51A*.

As the experimental sexual cross showed, the triple $TR_{46}{}^{3}$ can result from the sexual reproduction of two double TR_{46}. However, $TR_{46}{}^{3}$ Y121F/M172I/T289A/G448S may also have resulted from TR_{46} via subsequent mutations (Figure 10). In an evolutionary experiment it was shown that TR_{34} evolved to $TR_{34}{}^{3}$ under the exposure to tebuconaozle for 10 passages (Snelders et al. 2012). It is generally believed that microsatellite loci and the mating type are relatively stable via asexual and sexual reproduction. From this azole- containing compost, the microsatellite loci and mating type gene of $TR_{46}{}^{3}$ (V181-30)(V181-36) are similar to that of TR_{46} (V181-38), suggesting a possible clonal relationship. However, for the other two $TR_{46}{}^{3}$ (V181-39, 43), no matched TR_{46} type was found. These observations do not exclude a sexual origin of the isolates.

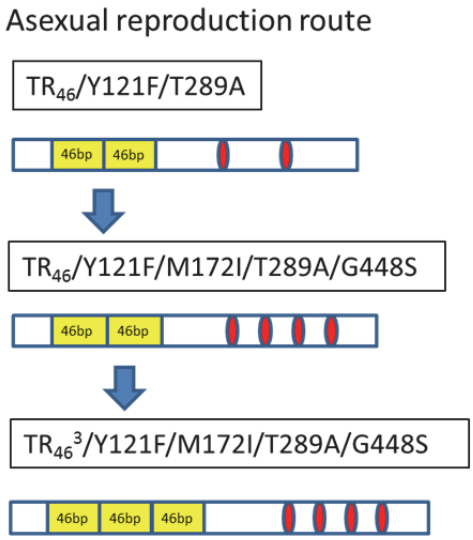


Figure 10. The possible origin of $TR_{46}{}^{3}$ /Y121F/M172I/T289A/G448S via asexual reproduction

Sex in the compost?

In our study, accumulating evidence suggests the possibility of occurrence of sex in the compost, such as:

1. Heat-shock treatment reduced the number of *A. fumigatus* colony-forming units significantly (Figure 3), suggesting that most, if not all, conidiospores were killed.
2. Isolates obtained after heat shock were genetically widely diverse, indicating that these were not derived from conidiospores from one or a few single clones (Table 1& 2).
3. Sexual crossing of two isolates with TR_{46} yielded (rare) progeny with the $TR_{46}{}^{3}$.

4. Two mating types of $TR_{46}{}^{3}$ were discovered from the same compost.

However, the lack of direct evidence of ascospores in the compost and the low-frequency survival of conidiospores to heat shock precludes the definite conclusion of the occurrence of sex in compost. In the future, diversity analysis before and after heat shock may be another way of testing for sexual reproduction in compost.

(1) Compost provides a favorable condition for sex

Several key factors for sexual reproduction under laboratory conditions that have been identified seem to be met in compost (O'Gorman et al. 2009). Compost as a multi-organic resource, with inside the heap a warm, dark, low oxygen/high CO2 environment as a result of organisms' activity, supplies all known essential elements for the occurrence of sex. *A. fumigatus* isolates of the two different mating types are widely spread in nature (Gow 2005). Combining these important elements, it is likely to find ascospores in compost.

In addition, it is believed that environmental changes affect the switching between asexual and sexual reproduction. It has been argued that in many fungi, sex occurs at the end of the growing season, when the conditions for somatic growth become adverse, at this moment, the cost of sex is low (Chamberlain et al. 1997; Hoekstra 2007). Hence, a dynamic composting process with temperature gradients (20-70°C) and gas changes might provide the suitable environment to sex when the cost of sex is low.

(2) Sex could provide benefits for the survival of *A. fumigatus* in the compost

1) Sexual reproduction can generate resting ascospores with survival structures (thick wall) to cope with unfavorable conditions.

Many planktonic organisms produce 'resting' stages by sexual reproduction when the environmental conditions deteriorate, such as Daphnia (Alekseev and Lampert 2001). In plants seeds can survive unfavourable conditions as well (Maynard-Smith 1978). In fungi, the outcome of sexual reproduction most often is the production of resting spores that are used to survive in extreme condition and to spread, for example *Plasmodiophora*, *Neurospora*: the dormant ascospores of *Neurospora* are activated by fire, and then start to germinate (Macfarlane 1970) (Henney and Storck 1964). All resting spores are thickly encysted in order to survive through stressful times. In *A. fumigatus*, the sexual ascospores have a thick cell wall and have the capacity to survive adverse condition, while asexual spores are destined to germinate quickly or act as fertilizing agents (spermatia) (Chamberlain and Ingram 1997; Hoekstra 2007; Kwon-Chung and Sugui 2013)). Related to this ecological specialization is the tendency of sexual reproduction to be induced when the environments are harsh. In response to the dynamic compost environment, *A. fumigatus* could increase survival rate via sexual reproduction.

2) Sexual reproduction generates genetically variable genotypes via recombination

It is known that sexual reproduction produces genetically variable offspring due to recombination between all alleles, sex also can speed adaptation and eliminated the deteriorate mutation much more efficiently compared with asexual process (Bell 1982; Camps et al. 2012; Losada et al. 2015). Schoustra has shown that the different genotypes of ascospore achieved highest fitness in different environments in *A. nidulans* (Schoustra et al. 2010). Therefore, highly diversed ascospore enhances the survival rate of ascospore and maximizing success in the later when the surroundings altered.

3) Sex can quickly generate resistance genotypes

Via sexual recombination, the resistant allele will be re-shuffled and generate the new variable resistance genotypes that may be better adapted to the changing azole environment either in the azole-exposed environment or in the patients. As we found in the sexual cross between two resistant TR_{46}, the promoter could extend into $TR_{46}{}^{3}$ and lead to increased resistance to voriconazole, itraconazole and posaconazole. Therefore in any environment with azole pressure the favorable genotype will be selected successfully.

Conclusions and Future Outlook

Our study shows that azole-exposed compost can serve as a hot spot that facilitates the spread of azole-resistant *A. fumigatus*. Although we found evidence that sexual reproduction might take place in the environment, we were unable to provide definitive proof. Nevertheless, we believe that the full life cycle of *A. fumigatus* needs to be taken into account to explain the emergence of azole resistance. The continued emergence of azole resistance mutations warrants research into the mechanisms of resistance selection in the environment. Insights in key factors involved in the selection or spread of resistance in *A. fumigatus* will help to develop strategies that prevent resistance selection. Only then can this important class be rescued for use for crop protection as well as treatment of *Aspergillus* diseases.

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CHAPTER VI

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Supplementary data

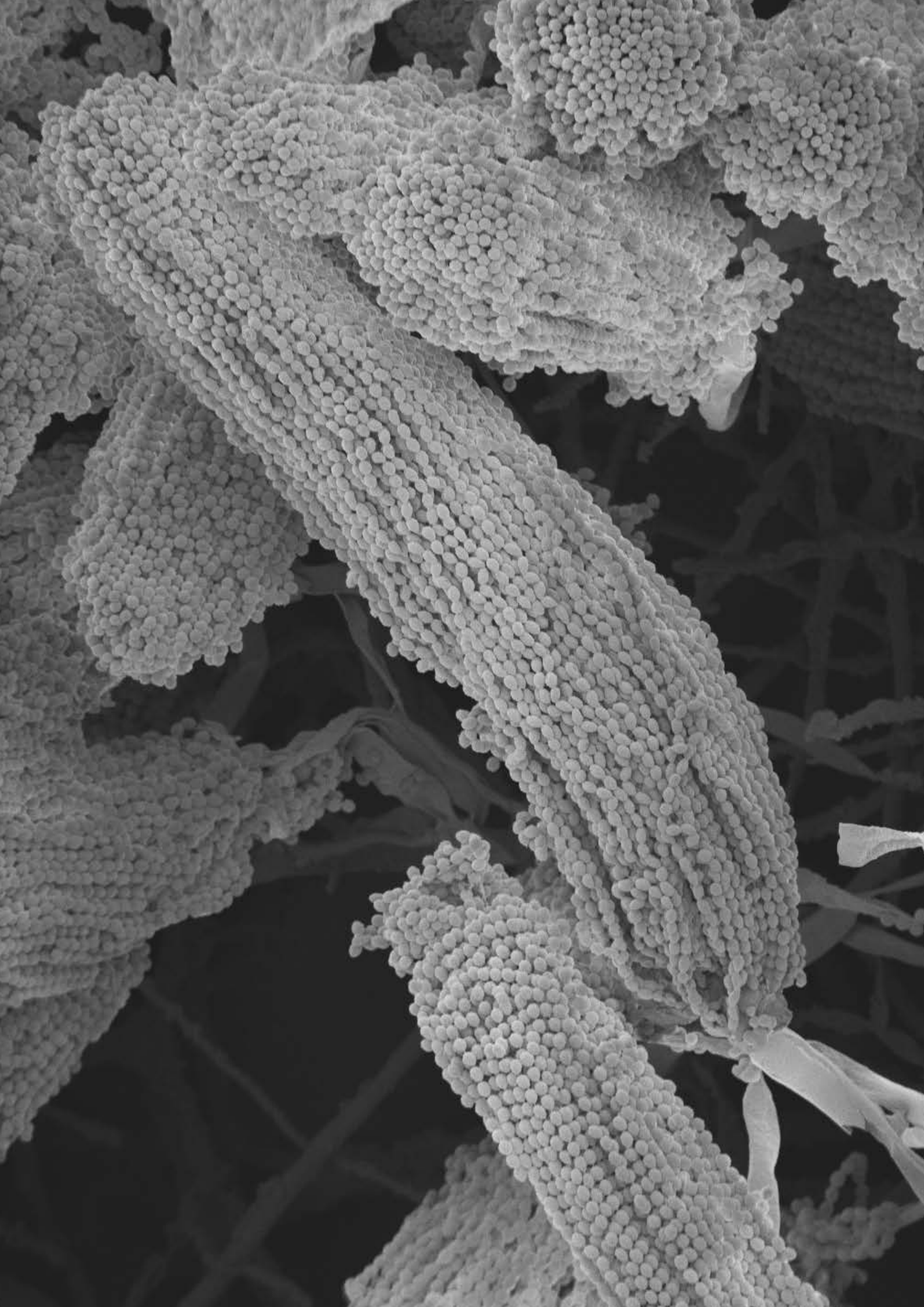
1. Fungicides detected from the azole-free Compost W and azole-containing Compost H

Samples	Detected fungicides
W1, 2, 3, 4, 5, 6	None
H1, 2, 3, 4, 5, 6	prochloraz 0.020 mg/kg
	prothioconazole-desthio 0.25 mg/kg
	imidacloprid 0.013 mg/kg
	boscalid 0.026 mg/kg
	tebuconazool 0.055 mg/kg
	prothioconazole-desthio 0.068 mg/kg

Methods:

The homogenized samples are weighed into a Teflon jar. A (homogenized) sub-sample is extracted with acetone, followed by extraction with dichloromethane / petroleum ether. A portion of the extract is evaporated to dryness, re-dissolved.

Re-dissolving is dependent on the analysis that follows. For the GC-MSMS (liquid chromatography-mass spectrometry) analysis, the sample is re-dissolved in iso-octane / toluene (9: 1). For the LC-MSMS (gas chromatography-mass spectrometry) analysis, the sample is re-dissolved in methanol redissolution acidified by 0.02% HAC. The content of ethephon, dithiocarbamates and other single residue methods are determined following the requirements of Euro Finnish.



CHAPTER VII

General Discussion

This thesis describes the role of several aspects of the ecology and lifecycle of *Aspergillus fumigatus* in the development of resistance to the azoles in its environment. Understanding the ecology and evolutionary dynamics of *A. fumigatus* is very important in order to make the right decisions in controlling the recent increase of azole resistance in clinical practice.

Relevance of the life cycle of *A. fumigatus* for the emergence of azole resistance

The various parts of the *A. fumigatus* life cycle are vital to generate genetic variation by mutation and recombination during mitotic and meiotic divisions. For this thesis I researched how these components of the life cycle relate to the emergence of azole resistance. Asexual reproduction (Figure 2, **Chapter 1**) is abundant in nature and occurs in patients with cavity aspergillosis as well. Under laboratory conditions, a single spore can form a colony with $5*10^9$ conidiospores within one week of growth. Each of these spores is the result of a mitotic division in one of the approximately 100 conidiogenous phiallide cells per conidiophore. Each phiallide may divide up to 100 times to create strings of conidiospores with up to 10^4 spores per conidiophore. Under the assumption of a genome-wide single-nucleotide mutation rate of $1\text{-}2*10^{-10}$ per base per mitotic division, as found in other ascomycetes (Lynch et al. 2008), and a genome size of 30 Mb, a one-week colony of *A. fumigatus* is expected to have more than 10^7 SNP mutations. In **Chapter 3** we provide the experimental evidence for the idea that asexual sporulation promotes the emergence of azole resistance by providing ample mutation supply (Zhang et al. 2015b). However, within and between strain variation in mutation rate during the sporulation process and mycelial growth is still unknown and needs further investigation in *A. fumigatus*.

For **Chapter 4** we sequenced 11 isolates with different resistance levels and morphologies from our evolutionary experiment that is described in **Chapter 3**. Evolved lineage D1, with increased azole resistance, contains two fixed mutations that have been confirmed to be involved in azole resistance by analyzing the progeny of a sexual cross. One of these azole-resistance mutations is a known CYP51 gene mutation; the other is HMG CoA reductase mutation, a rate-controlling enzyme of the mevalonate pathway that produces ergostrol. The finding that the evolutionary lineages without azole treatment also harbour mutations is consistent with the idea that asexual sporulation generates genetic diversity via mitotic division. Whether these mutations are neutral or are related to adapting to the specific experimental conditions is not clear.

Sexual reproduction starts with fusion of two strains of different mating types. New genotypes are generated via recombination of the parental genomes as a result of crossing over and independent assortment of chromosomes during meiosis (Figure 2, **Chapter 1**). In **Chapter 3** we show that the sexual cross between evolved lineage D1 and a sensitive isolate, generates four genotypes, as expected from the independent

segregation of the two resistance mutations by meiotic recombination. Sexual reproduction could speed up adaptation via recombination by creating variants with increased azole resistance. We have support for this now from data that show the generation of highly–resistant progeny from a cross of intermediate resistant and sensitive isolates (Figure 1). Furthermore, the new resistant genotype TR_{46}^{3} is generated from a sexual cross of two TR_{46} parents, presumably via unequal crossover during meiosis. These results provide evidence that sexual reproduction promotes the emergence of new azole-resistance mechanisms. However, details of meiosis in *A. fumigatus,* such as the recombination rates, unequal crossover, and the expansion of repeats or gene copy variation need further investigation. In addition, the contribution of *de novo* mutations during sexual reproduction is unknown.

So far, sexual reproduction has only be observed under strict laboratory conditions (O'Gorman et al. 2009). The data shown in **Chapter 6** suggest the potential occurrence of sexual reproduction in nature. However, when, where, and in what frequency sexual reproduction occurs in nature needs a more in depth study. If sex occurs in nature, sexual reproduction may explain the high diversity of microsatellite genotypes in globally collected isolates.

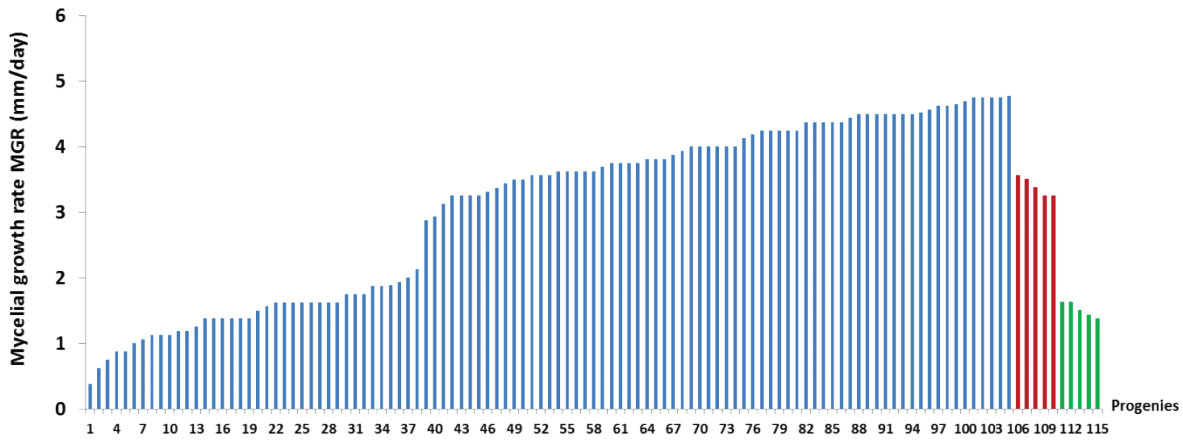


Figure 1. Segregation of mycelial growth rate (MGR) phenotypes among the progeny from a sexual cross between the evolved strain (D3 fluffy, parental type 1; P1; with high MIC and fitness) and a sensitive genotype (parental type 2; P2).

MGR of progenies was measured on the MEA containing 1 µg /mL difenoconazole. Columns 1-105 represent individual progeny, 106-110 represent parental type 1, evolved strainD3-5 fluffy (red), and columns 111-115 represent parental type 2, sensitive environmental isolate (green). Note that some progeny (67-103) have higher fitness compared to the resistant parents P1.

The parasexual cycle includes mycelium growth, diploidization via nuclear fusion, and haploidization into haploid again. Since the somatic rate of diploidization is relatively low with a frequency around 10^{-5} (see **Chapter 3**), recombination via the parasexual cycle is expected to be limited. However with the large number of mitotic divisions occurring during somatic growth and sporulation it is still possible that aspects of the

parasexual processes play a role in speeding up the evolution of azole resistance via recombination.

For the development of resistance asexual reproduction seems most relevant in generating SNP mutations through large numbers of mitotic divisions, while the (para)sexual part of the lifecycle are more likely to generate structural variation such as duplications and recombined combinations of existing mutations via meiosis and mitotic recombination.

Do azoles influence the evolution of azole resistance in other ways than direct selection pressure?

Our environment is contaminated by azole compounds due to a wide variety of usages: azoles are applied in shampoo, for wood preservation, in wall paper, and on agricultural crops for protection (Verweij et al. 2009; Gisi 2013). In addition, bacteria can produce azole-like compounds (Łukowska-Chojnacka et al. 2016) a so far neglected aspect with respect to azole-resistance development in fungi, and also not addressed in this thesis. All these azoles pose selective pressure on the widely spread *A. fumigatus.*

In this thesis I have shown that azole exposure to *A. fumigatus* will provoke a strong selection for azole resistance. The experiments in **Chapters 3** and **4** have shown that difenoconazole exerts the strongest selection pressure compared with other fungicidal azoles. This can be explained by the molecular structure of these azoles and their binding sites when they interact with fungal CYP51A.

In **Chapter 6** I confirm that also in the environment azoles create strong selection pressure. The striking contrast between *A. fumigatus* isolates from azole–containing composts and from azole-free compost strongly suggests the vital role of azole selection. Whereas in the azole–containing compost most *A. fumigatus* isolates were azole-resistant (92%), in the azole-free compost most isolates were sensitive (98%).

Different azoles select different genotypes of *A. fumigatus*, as we can see from Figure 2. From the same compost sample containing three different genotypes, wildtype, TR_{46}/Y121F/T289A, and $TR_{46}{}^{3}$/Y121F/M172I/T289A/G448S, on petri-dishes containing the agricultural fungicide difenoconazole, all three genotypes are able to survive, which may be explained by pre-adaptation in the compost contaminated with agricultural fungicide. However, if they encounter the medical azole itraconaozle, a novel environment, only the TR_{46}/Y121F/T289A genotype can survive. This information underpins the dominant genotype in the clinical isolates rather than environmental isolates due to the second selection of medical azoles.

There may be additional influences from azoles on the evolution of azole resistance. Azoles are not known to be mutagenic or recombinogenic, however, azoles may speed up the adaptation of *A. fumigatus* by posing (physiological) stress. For instance, azoles slow down growth and disrupt the homeostasis in the cell which may negatively affect

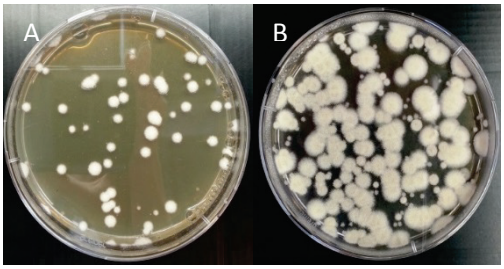


Figure 2. The response to agricultural and medical azoles of three *A. fumigatus* genotypes from a natural sample of compost.
A; Only TR_{46}/Y121F/T289A is capable to grow on medical azole itraconazole (32g/mL); B: Wild type (small colonies), $TR_{46}{}^{3}$/Y121F/ M172I/T289A/G448S (intermediate colonies) and TR_{46}/Y121F/T289A (big colonies) grow on agricultural azole difenoconazole (32g/mL).

DNA-repair mechanisms and general "quality-control" of spores which will increase the mutation frequency. Moreover, it has been discussed that sex occurs as a reaction to stressful conditions (Chamberlain et al. 1997; Hoekstra 2007). Azole might be a stress signal for *A. fumigatus* to go through sexual reproduction to generate more diverse genotypes to cope with stressful or changing environment. Therefore it is possible that azole in the environment speeds up the rate of evolution in *A. fumigatus* through sex. Therefore it is important to study the role of azole and other stress factors on the induction of sex in *A. fumigatus*.

Azole-resistance development routes: Environmental or clinical routes, or both?

Azole resistance in *A. fumigatus* is increasing over the years, and especially the mutants TR_{34}/L98H and TR_{46}/Y121F/T289A have spread globally. To answer when and where these types of resistance mechanisms first emerged has become an important puzzle in order to find a solution for this increasing problem (Snelders et al. 2008; Chowdhary et al. 2012; Ahmad et al. 2014; Astvad et al. 2014; Rocchi et al. 2014; Pelaez et al. 2015; Snelders et al. 2015; Wiederhold et al. 2015).

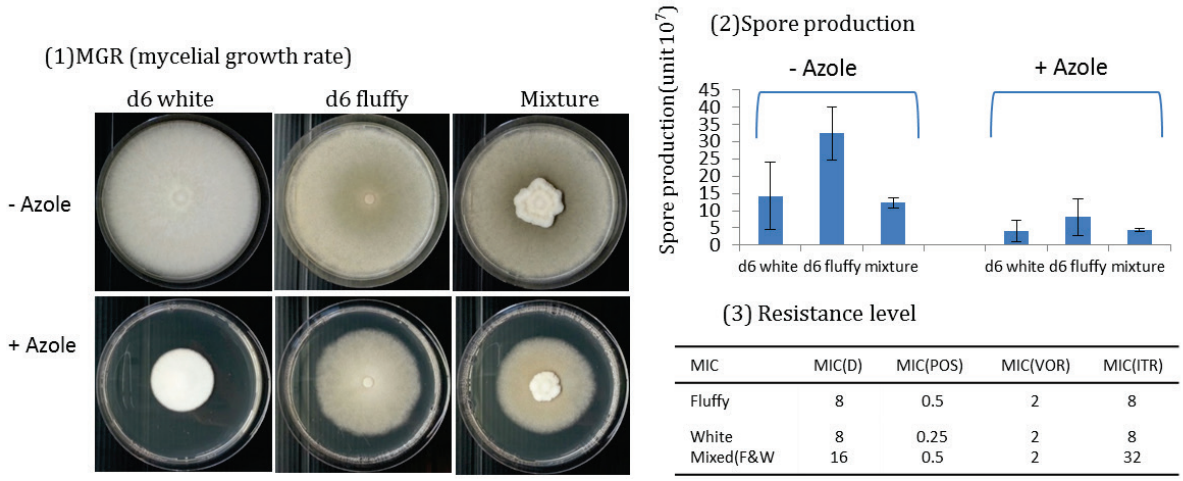
Resistant isolates can be collected from patients with chronic aspergillus disease, such as CF patients who are under prolonged treatment with medical azoles and from evolutionary experiments with around seven weeks of exposure to environmental fungicides (Camps et al. 2012b; Zhang et al. 2015). **Chapter 4** describes that the evolutionary patterns of evolved lineages to agricultural azoles are highly correlated to the pattern of the medical azoles, which indicates the possibility of an environmental route to resistance development. However, the frequently found azole-resistance mechanisms containing TR_{34}/L98H and TR_{46}/Y121F/T289A have not yet been discovered from any evolution experiment with various azole types. Interestingly, CYP51-G138S mutants are reported both from the patients with long-term azole treatment and from an evolutionary experiment as well, which supports the possibility of both azole resistance development routes (Snelders et al. 2012).

Considering the relevance of all aspects of the life cycle in *A. fumigatus* in the emergency of azole resistance, it seems that environmental routes have more opportunities to facilitate the development of resistance. *A. fumigatus* can produce enormous numbers of asexual spores in the environment (Zhang et al., 2015) and it is likely that it can complete the whole life cycle in nature although direct evidence for sexual reproduction is still lacking. However, in patients, a totally different environment, the fungal biomass will be limited and mostly consist of mycelium, and asexual reproduction will occur only in cavitary aspergillioma. Parasexual recombination might occur in patients with a long-lasting infection, but sexual reproduction is highly unlikely in patients. So the wide use of azole compounds in the environment, the prevalence of *A. fumigatus* in decaying vegetation and no restrictions for completing its lifecycle make it likely that the environmental route contributes mostly to the recent increase of azole resistance. However the clinical route may also contribute to azole-resistance development, especially when asexual sporulation can occur and infections are persistent and lasting for many years. Clearly more research is needed to assess how resistance can develop in patients and in the environment and how these resistant types can spread.

It is known that compost is an important ecological niche of *A. fumigatus*. Compost houses a dense community of various fungi and bacteria which have to compete for their share of the resources. Some bacteria such as *Burkholderia andropogonis*, *Pseudomonas chlororaphis* are able to produce azole types of compounds, such as 1H- imidazole, 1-ethyl- 5-Cyano-1,2,3- thiadiazole and Banzothiazole (Łukowska-Chojnacka et al. 2016). It is possible that *A. fumigatus* is exposed to these natural azoles and has acquired resistance as well. Whether this also affects resistance to other azoles is unclear. If *A. fumigatus* has adapted to these azole-like compounds in the compost during its evolutionary history, it is not surprising that *A. fumigatus* already has flexible defense mechanisms that can quickly develop resistance to new medical azoles. However, how and which type of azole-producing bacteria might contribute to the recent increase in azole resistance of *A. fumigatus* need further study.

Morphotypes of *A. fumigatus* in the environment and patients

As shown in **Chapter 5**, several different morphotypes evolved during the evolutionary experiment in the laboratory, with changes of colony size, mycelium structure, and early or late sporulation. The dynamic morphology changes of *A. fumigatus* over the azole treatment suggest that morphology is an important factor for *A. fumigatus* during adaptation to the azole environment. Cooperation between morphotypes and negative interactions such as cheating may also contribute to adaptation to the azole environment at the population level. We have shown such interaction between coevolved morphological types in both lineage D3 and D6 that have higher resistance levels when growing together, but have reduced spore production in the D6 lineage (**Chapter 4** and Figure 3). Possibly their morphologies evolved in order to optimize their interactions with the type they evolved with. Notably, the earlier found correlation between MIC value and MGR does not seem to apply for morphological mutations (e.g. see D6 white and D6 fluffy, Figure 3).



MIC	MIC(D)	MIC(POS)	MIC(VOR)	MIC(ITR)
Fluffy	8	0.5	2	8
White	8	0.25	2	8
Mixed(F&W	16	0.5	2	32

Figure 3. Interaction between d6 white and d6 fluffy in the present and absent of azole environment

The mixture has lower mycelial growth rate (MGR) and spore production, however, exerts a higher resistance level in the presence of azole.

Most clinical isolates collected from patients with different aspergillus infection also show morphological variation (**Chapter 5**, Table 2). In some cases, non-sporulating *A. fumigatus* isolates are found from clinical patients (Zhang et al. 2015a), which is very different from the natural environment with decaying plant material. The human environment clearly differs by a low Fe, low O_2, different nutrients, temperature and light conditions as well as an immune system. Loss of sporulation after a long-stay inside a patient, may reflect a pleiotropic effect of genetic adaptations to the human body and/or medical azole treatment.

Although it seems that morphology is important for azole resistance in *A. fumigatus*, generally its role in developing resistance to azole has been neglected and deserves more attention.

The possible origin of TR_{34}/L98H and TR_{46}/Y121F/T289A and TR_{46}^{3}/Y121F/M172I/T289A/G448S

The pre-dominant resistance genotypes found in *A. fumigatus* isolates from the Netherlands have a combination of tandem-repeat (TR) variations in the promoter region and point mutations in the open reading frame of the *cyp51A* gene. The origin of a TR is unclear, but it may be due to a replication error. The point mutations probably occur during mitotic division in the asexual sporulation structures of *A. fumigatus.* The spontaneous emergence of two genetic changes is highly unlikely to occur at the same time in the same individual and thus one must have been selected first before the other one occurred. It is tempting to speculate that the initial point mutation(s) in the *cyp51A* gene reduced the blocking affinity of azoles to the CYP51 enzyme as well as reducing the activity of the enzyme. The TR in the promoter would than compensate for the reduced enzyme activity by upregulating the *cyp51A* gene.

Genotype analysis based on microsatellite loci shows enormous diversity in TR_{34}, TR_{46}, and $TR_{46}{}^{3}$ isolates, which makes it difficult to conclude the possible evolutionary route of these TR variants (Debeaupuis et al. 1997; Duarte-Escalante et al. 2009; Araujo et al. 2010). It is generally believed that the microsatellite alleles and mating-type remain unchanged via asexual reproduction, and even after sexual reproduction (just shuffling genes rather than changing), therefore it is difficult to explain the origin of highly diverse microsatellite loci. One possible explanation is that the microsatellite loci are not stable and influenced by many factors.

In **Chapter 6** I showed that $TR_{46}{}^{3}$ can be generated via a sexual cross between two TR_{46} with opposite mating type and isolated from the same compost sample, even with low frequency of 10^{-5}. This suggests a vital role for sexual reproduction in the development of azole resistance by increasing the tandem repeat number. However, the evolution of TR_{34} to $TR_{34}{}^{3}$ after 10 passages of experimental evolution (Sender et al, 2012) suggests that tandem repeat number can also increase via DNA duplication error in asexual reproduction.

Can *A. fumigatus* complete its life cycle in nature?

There are three main aspects of the life cycle of *A. fumigatus* that are relevant for fungal adaptation and evolution: asexual and sexual reproduction and the processes associated with the parasexual cycle. As mentioned before, each of these variations can potentially have their role in the development of azole resistance.

Asexual reproduction is abundant in nature, as we can see from the isolation of enormous amount of conidiospores from compost, soil, air, and even in the office (Anastasi et al. 2005; Deacon et al. 2009; Gisi 2013; Kwon-Chung and Sugui 2013; Anastasi et al. 2005; Deacon et al. 2009). Asexual reproduction is a very efficient and essential way of (1) dispersal for *A. fumigatus* due to the large number of conidiospores produced and their airborne character, (2) alternation from the multinuclear hypha to uninuclear conidiospore and further form the homokaryotic cultures, and (3) of mutation supply.

Sexual reproduction in *A. fumigatus* was discovered in 2009, and has thus far only been demonstrated under specific and strict laboratory conditions (dark, 30°C and oatmeal medium with two opposite mating-type strains). The enormous genetic diversity in the natural *A. fumigatus* population however suggests the occurrence of sexual reproduction, but whether sexual reproduction really occurs in nature remains an open question. As **Chapter 6** has shown, compost seems a favourable condition for the occurrence of sex. However, when and where, and under which condition exactly sex can occur in nature is still unknown. Whether our method with heat shock to distinguish the asexual conidiospores and sexual ascospores is a reliable method needs further investigation, before we can conclude whether sexual reproduction has taken place in compost.

Parasexual or somatic recombination starts at the hyphal stage with heterokaryotic mycelium formed by two compatible isolates or upon spontaneous mutation in the

hypha. Next heterozygous diploid nuclei can be formed; crossing-over and nondisjunction subsequently result in recombinant haploids. Heterokaryon formation by spontaneous mutation in somatic nuclei is likely to happen during mitotic division and may be especially relevant in long-lived cultures. Heterokaryons can also result from anastomosis between different hyphae. However, fungi have an extensive self-non-self recognition system preventing such heterokaryon formation, so that two random isolates are almost always heterokaryon incompatible. Whether heterokaryon incompatibility is also widespread in *A. fumigatus* is so far unknown, but highly likely. In **Chapter 3**, it was shown that diploids could be isolated under laboratory condition with a frequency of approximately 10^{-5} among spores from a heterokaryon. However, whether diploid formation and subsequent haploidization is able to occur under the conditions in nature requires more in-depth study. Another possible cause of diploid formation is sexual reproduction. In *A. nidulans* it was reported that approximately 1% of the ascospores is diploid (Pritchard et al. 1954). For *A. fumigatus* this has not been studied, but if this also applies to *A. fumigatus* a relatively high frequency of diploid isolates would be expected if sexual reproduction is abundant in nature.

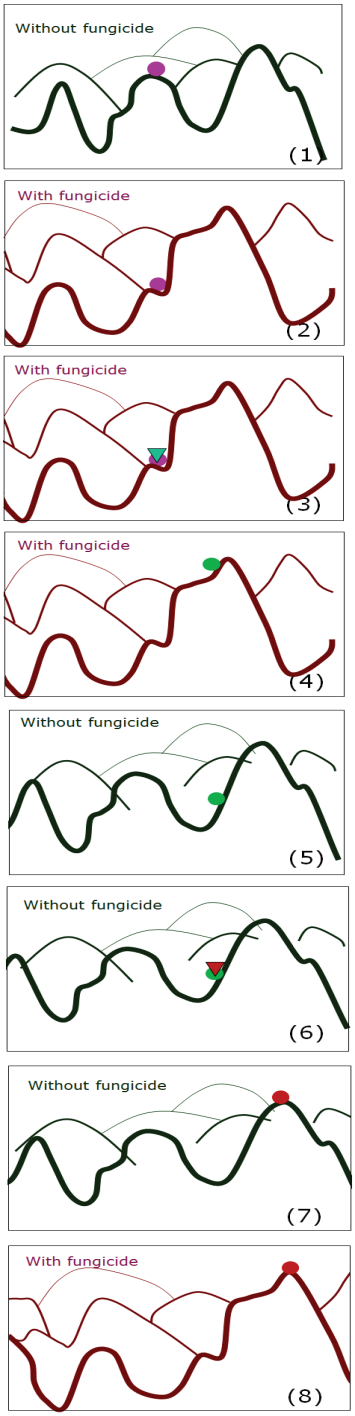


Figure 4. Development of resistance and subsequent compensatory mutations in the context of adaptive landscapes. Panel (1) shows a stable situation, the isolate is on a fitness peak. Introduction of a fungicide, changes the landscape which lowers the fitness of the given genotype [Panel (2)]. A mutation (green sector) leading to higher fitness can occur [Panel (3)] which results in an adaptive walk to the new fitness peak in the fungicide environment [Panel (4)]. Changing the landscape again by removing the fungicide [Panel (5)] makes that the resistant isolate is no longer on a fitness peak and mutations [Panel (6) red sector] will be selected that compensate for this loss of fitness. The resistant isolate with compensatory mutations approaches a fitness peak that is high in either absence or presence of fungicides [Panels (7) and (8)]. The figure is adapted from (Schoustra et al. 2006). Colour dots: different adaptive genotypes; X-axis: the appearance of different genotypes over the evolutionary time; Y-axis: fitness such as MGR. Green landscape: environment without fungicides. Red landscape: environment with fungicides.

Compensatory mutation leads to persistent azole resistance in *A. fumigatus*

It is believed that resistance always brings along a fitness cost, in particular when a resistant strain is growing in the absence of azoles. This cost can for example be

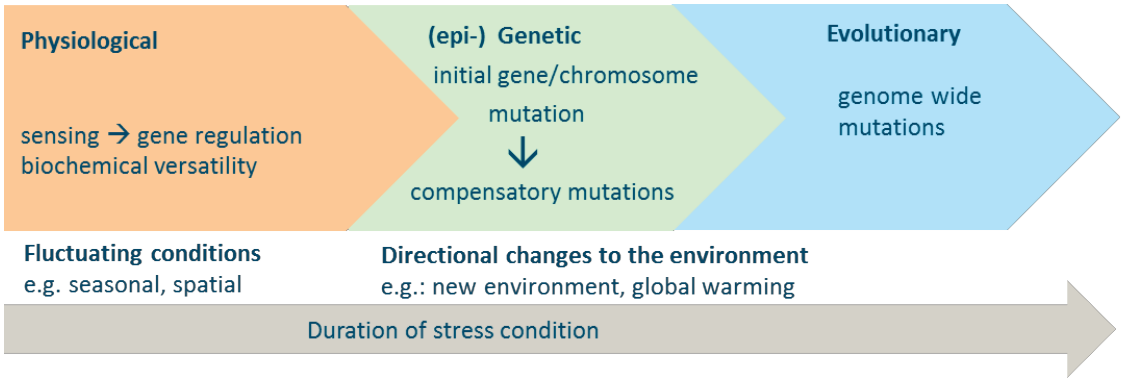
observed as reduced mycelial growth rate compared with a wild-type strain (**Chapter 1**, Figure 2). Therefore, resistant isolates might lose their advantage over wild-type isolates when they compete in the absence of azole. This cost would severely restrict the spread of resistant isolates, however, the gain of compensatory mutations eliminates this cost for the resistant isolate and it can reach the same fitness as the wild type in an azole–free environment while keeping its azole resistance. How a compensatory mutation can increase the fitness is shown in figure 4. The possibility for compensatory mutations will make controlling the spread of azole-resistant isolates more difficult as reducing the use of azoles will not necessarily lead to the disappearance of resistant isolates already present in the environment. Therefore it is important to understand the mechanism of compensatory mutations.

The adaptation of *A. fumigatus* to the environment/ human host

Adaptation is the process in which organisms improve their fitness in a given environment by changing their phenotype using genetic or non-genetic mechanisms. Different adaptations are characterized by the time needed for organisms to attain them and by the duration of the stress condition. The fungal adaptation involves physiological, (epi-) genetic and evolutionary adaptation (Figure 5A). *A. fumigatus* is a biochemically versatile organism capable of growing under a wide range of conditions (e.g. pH, temperature, nutrient source) based on sensing and according gene regulation. This is an adaptation to its lifestyle: the airborne conidiospores may land upon unpredictable substrates and will thus have to be able to cope with a diversity of environmental conditions. Phenotypic plasticity can potentially ensure survival and reproduction under many conditions and therefore may contribute to the fitness of an opportunistic fungus. *A. fumigatus* has e.g. 22 secondary metabolite genes and high number of efflux pumps to adapt to fluctuating condition (Sugui et al. 2007; Buied et al. 2013; Fraczek et al. 2013) Figure 5B shows *A. nidulans* is capable to grow on a wider range of pH by coordinating the multi- secondary metabolite genes, such as changing the colony colour, rather than alkaliphilic fungus *sodiomyces alkalinus,* only is able to survive at the high pH environment. However, physiological adaptations are generally not inherited over generations. (Epi-) genetic adaptation may occur at the DNA (mutation or copy number variation) and chromosome, RNA, and even protein level, when there are longer-lasting directional changes to the environment (Yona et al. 2015), e.g. with widespread presence of azoles. Such conditions may explain the emergence of genetic adaptations such as the cyp51 mutations (G54E and TR_{34}/L98H), and the transcription factor hapE mutation in *A. fumigatus* (Snelders et al. 2010; Albarrag et al. 2011; Camps et al. 2012a; Kwon-Chung and Chang 2012). Genetic adaptations are generally memorized and can be propagated to the next generation. However, so far no evidence yet for epigenetic changes are involved in azole resistance. With extended duration of directional changes, genome wide mutations may occur, e.g. to compensate for cost of resistance effects, and lead to gradual evolutionary changes due to the stress as previously discussed in other species (Zong et al. 2012; Long et al. 2016). The combined effect of several mutations across the whole genome might underpin adaptation to directional changes.

As shown in **Chapter 4**, when the sensitive ancestor adapted to the azole environment in seven weeks, several phenotypes evolved during this adaptation process. In D1 lineages, the azole target gene *cyp51A* mutation was discovered, as a genetic adaptation that can be inherited to offspring by sexual or asexual reproduction.

A



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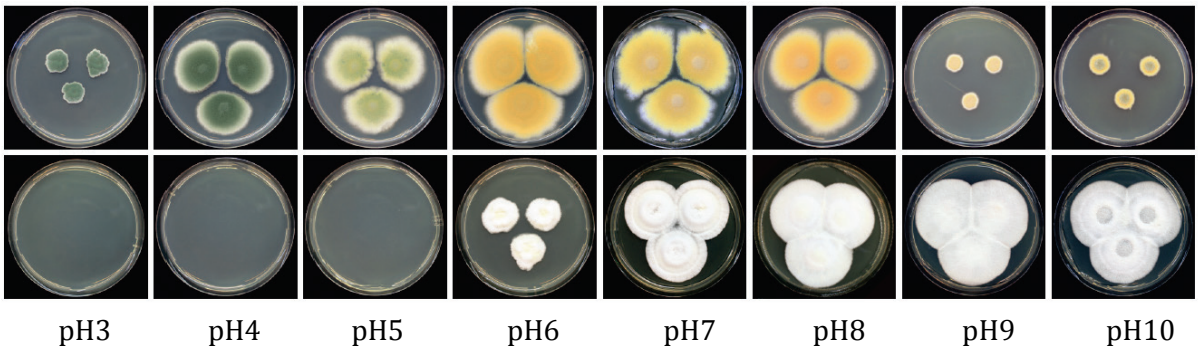

Figure 5. A: the *A. fumigatus* adaptation spectrum over the duration of stress condition: physiological, (epi)-genetic and evolutionary adaptations.
Physiological adaption is related to the fluctuating conditions such as seasonal or spatial changes. Whereas the (epi)-genetic and evolutionary adaptation fit the long –term of directional changes to the environment.

B: *A. nidulans* can grow on a wider range of pH, than alkaliphilic fungus *sodiomyces alkalinus* (obtained from Grzhimaylo, 2015).

In D6 lineages, two mutations were identified that were not present in the ancestor. The trace-back check showed that HPT (hpt-protein-AFUB) is highly likely involved in azole resistance, whereas the PTAB mutation is associated to the phenotypic change of later sporulation (white phenotype). This is a typical evolutionary adaptation case in azole-resistance development.

Hotspots for azole-resistant A. *fumigatus*

A. fumigatus has been found in patients, agricultural fields, flower-beds, compost, air, sewers, and even in bathrooms. However, understanding what the hotspots for azole-resistance development and maintenance are, is crucial for more efficient azole-

resistance management. In **Chapter 6** we showed that the azole–containing compost is a potential hotspot for azole-resistant *A. fumigatus.* Although the azole concentration we found in this compost is not high as Gisi mentioned (Gisi, 2013), it can still provide the effective selection for azole-resistant *A. fumigatus.* At the same time, this low concentration will not completely inhibit the growth, asexual reproduction, and potential sexual reproduction of *A. fumigatus*, so that resistance can develop over long periods.

As shown in **Chapter 6**, the sexual cross between two strains with TR_{46} produces more fruiting bodies (cleistotecia) on medium with a similarly low amount of agricultural azole compared with azole-free medium. This confirms the vital role of azole in the maintenance of resistance and further resistance development.

Overall, effective selection of resistant *A. fumigatus* can occur if (1) the fungus is able to grow with asexual sporulation or sexual reproduction and (2) there is an effective azole concentration as selection pressure. These two conditions appear to be crucial for the definition of what is a hotspot for the development of azole resistance. Thus, compost heaps contaminated with azoles and *A. fumigatus* infected patients treated with azoles are both potential hotspots. Extensive sampling for *A. fumigatus* on places where azoles are used or azole contamination can be expected will have to be done to reveal all potential hotspots for resistance development.

Can *A. fumigatus* become a real human pathogen?

It is generally believed that *A. fumigatus* only infects immunosuppressed people, such as patients with HIV. However, it has been found that a healthy dolphin can be struck with an *A. fumigatus* infection with the TR_{46}/Y121F/T289A resistance mechanism (Paul Verweij, personal communication). Therefore it is uncertain whether *A. fumigatus* can still only infect immunosuppressed people or it is maybe on its way to become a real human and animal pathogen.

If *A. fumigatus* can persist in the human body for a long time, it may gradually adapt to this new environment and become specialised and evolve into a true pathogen. Especially when the fungal adaptations are not an evolutionary dead-end but can "escape" from a patient to the environment via the dead body or to other patients via cough. Asexual sporulation is known to increase general mutation supply and strongly contributes to adaptation, and subsequently may lead to azole-resistance development (Zhang et al. 2015b). But it may also lead to dispersal of human-adapted genotypes. The current increasing numbers of patients with chronic aspergillosis that are difficult to treat and therefore have fungal growth and selection pressure for an extended period of time in the patient, may therefore pose a serious problem, at least from an evolutionary perspective.

Conclusion and future directions

In this thesis, I have demonstrated that the evolution of azole resistance is complex as the fungus has different ecological niches and a complex lifecycle and all the factors associated with this may affect the different aspects of azole-resistance evolution. Furthermore, facing up the current dilemma for the treatment of aspergillus infections, neither stopping the azole treatment nor changing to a new azole is the ideal solution. Therefore, the various aspects of the life cycle and azole-selection pressure need to be taken into account for understanding the development of azole resistance in *A. fumigatus*. To control the increasing amount of azole resistance and persistence of azole resistance in *A. fumigatus*, it is essential to understand all the details of the life cycle and ecology of *A. fumigatus* and interaction between both before we take actions to control the worldwide increasing problem of azole resistance in the further study.

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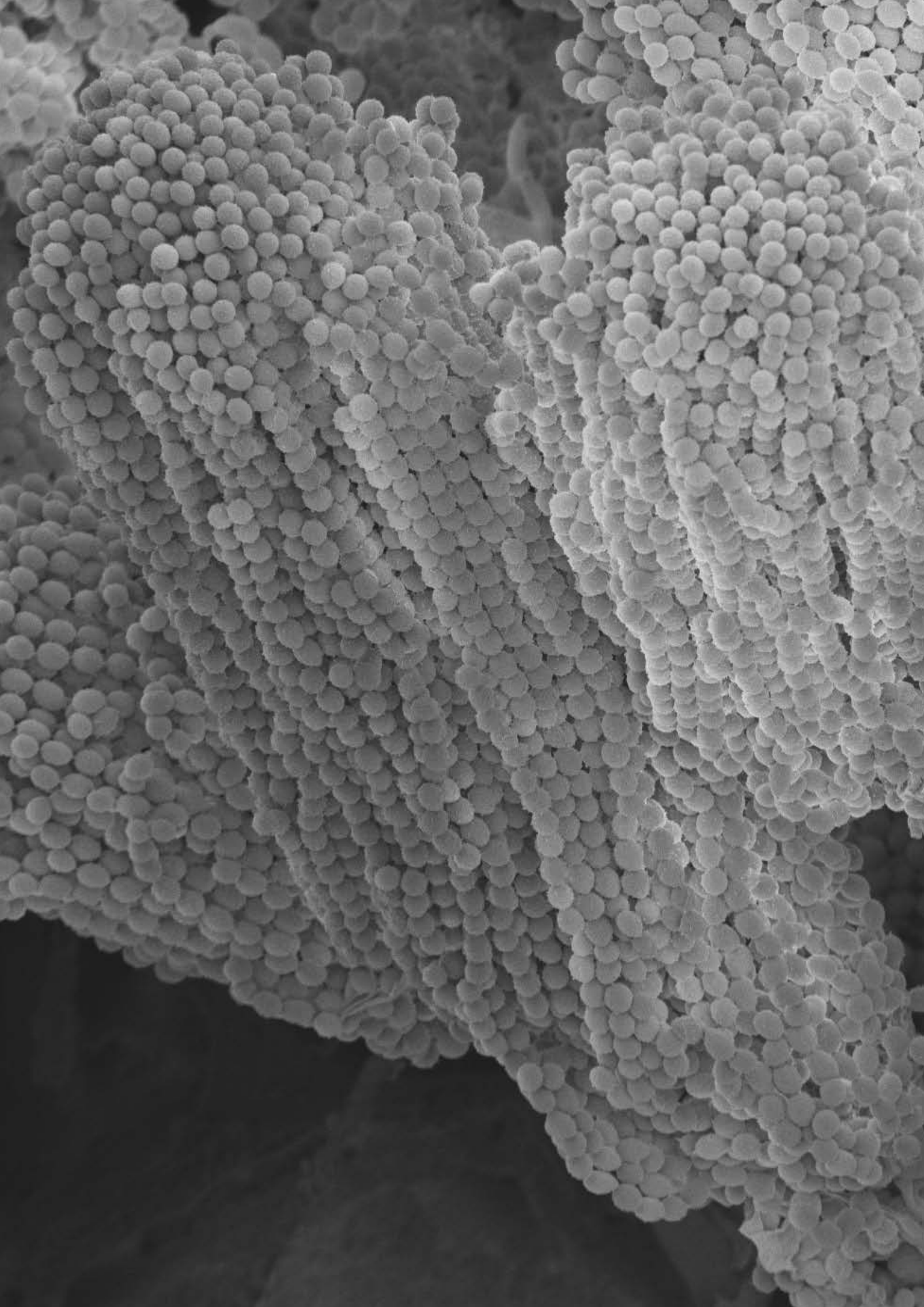

Summary

The presented thesis shows the relevance of the life cycle of *A. fumigatus* to the development of azole resistance and possible evolutionary routes that lead to it. The work highlights the importance of fungal biology and evolution towards understanding the development of azole resistance in fungi. I conclude that azole resistance in *A. fumigatus* is a consequence of selection pressure by azole in the environment on the genetic variation generated via various aspects in the *A. fumigatus* life cycle. This thesis also introduces an experimental evolution approach to study the dynamics and mechanisms of the evolution of azole resistance. In addition, I investigate what condition can lead an environment to be a possible hotspot for the development of resistance. Finally, I link this to the potential conditions under which resistance can emerge and spread in the lungs of humans and how this depends on the specific azole used.

The introduction in **Chapter 1** provided detailed information on the azole resistance in *A. fumigatus.* Azole resistance in *A. fumigatus* has become a global issue and hinders the efficiency of azole treatment in the clinical practice of aspergillosis infections. It is generally believed that there are two possible azole-resistance development routes with a different origin of the resistance: the clinical and the environmental route. That means azole resistance may develop in patients treated with azole creating a selective pressure, but azole resistance may also occur in the environment before *A. fumigatus* comes in contact with the patient. Different azoles used in agriculture can potentially lead to resistance to medical azoles. The major resistance mechanism is the alternation of the azole target gene *cyp51A*, encoding a key enzyme in the ergosterol pathway. The prevalence of highly resistant TR_{34}/L98H and TR_{46}/Y121F/T289A has increased over the recent years and spreads globally. It is known there are different modes of reproduction in *A. fumigatus,* however, the complex life cycle has so far been neglected for understanding how azole resistance develops. Furthermore, direct observation of the development of azole resistance in environmental isolates over evolutionary time is still missing. Finally experimental setting has not been used to explore the variations of agricultural azoles in the selection pressure.

Chapter 2 discussed the current dilemma on the management of *A. fumigatus* infections in patients: stopping the use of clinical azoles when resistance arises or changing to new triazoles? These two solutions are generally acceptable for controlling the emergence of azole-resistant *A. fumigatus* strains. By stopping the use of clinical azoles, the resistant *A. fumigatus* may initially suffer from the cost of resistance due to the unfavourable free-azole situation. The selective pressure of this cost may then lead to compensatory mutations that cover the cost of resistance potentially resulting in a resistance genotype without any cost. Therefore, compensatory mutations, due to the intermittent use of the clinical triazoles maintain the azole resistance in *A. fumigatus*. Similarly, changing to a new azole will promote the development of more mutations and may result in pan-resistance to all clinical triazoles. In **Chapter 2**, we provided a patient case of azole-treatment failure. Upon azole treatment, cultures exert a variety of phenotypes (different resistance level and morphotypes). As the current two treatment solutions,

compensatory mutations or multiple mutations may arise and both possibly contribute to treatment failure against *A. fumigatus* infection.

This chapter raises our concern for managing azole resistance in the clinical practice. To tackle this problem, understanding by what mechanism(s) the resistance emerges, how it can spread, and how resistant genotypes can persist in environments without azoles are important issues.

In **Chapter 3,** we focused on the role of asexual reproduction in azole-resistance development. It was previously hypothesized that asexual sporulation is required for the expression of azole resistance in *A. fumigatus* (Verweij et al. 2009), because the asexual sporulation process aids to release the resistant nuclei from multinuclear hypha to single conidiospores, which can grow into a full resistant hypha. We performed an evolution experiment with and without asexual sporulation. We then showed that the cultures which went through asexual sporulation reached a higher resistance level compared to the cultures without asexual sporulation. We concluded that asexual sporulation plays an important role in emergence of azole resistance. Further, we demonstrated that azole resistance is not fully dominant, which fits with the hypothesis that asexual sporulation is required to aid the resistant nuclei escape from the multinuclear hyphae. In addition, we put forward another hypothesis that asexual sporulation could increase the mutation load due to the large number of mitotic divisions during asexual sporulation. The enormous amounts of asexual spores produced enhance the mutation load, and therefore promote the emergence of azole resistance. This discovery highlights the importance of a specific life cycle part that leads to the emergence of azole resistance in *A. fumigatus*. It may also explain the resistance increase during the treatment of cavity aspergillosis in patients, where asexual sporulation takes place. Furthermore, asexual sporulation may also increase the dispersal of *A. fumigatus*, explaining the wide spread of TR_{34}/L98H and TR_{46} /Y121F/T289A mutants.

In **Chapter 4**, we debate on the two possible routes of azole-resistance development (clinical and environmental routes). For this we performed an evolution experiment where we exposed the sensitive ancestor strain to five agricultural fungicides that have been used over the past 30 years. We checked whether we can get the prevalent TR_{34}/L98H resistance mutant or other highly resistant isolates. Furthermore, we investigated whether the selected resistant isolates had developed cross resistance to medical azoles as well. We showed that the dynamic evolution trajectories of resistance against medical and agricultural azoles have high similarity, strongly supporting the agricultural route hypothesis. Evolved isolates differed in resistance level, growth rate, colony structures and sporulation time. However no TR_{34}/L98H mutant was found after the evolutionary experiment, but we did find one mutation G138S on *cyp51A* in one out of 36 lineages. Our results provide the direct evidence of possible environmental route, consistent with the previous studies, where the highly resistant TR_{34}/L98H exerts the cross resistance between medical and agricultural azoles. However, it is not clear

whether agricultural azole is capable of inducing the TR_{34}/L98H and/or TR_{46} /Y121F/T289A.

In **Chapter 5**, we studied the relevance of the heterokaryotic state in the development of azole resistance in *A. fumigatus*. A heterokaryon is a mycelium that contains a mixture of genetically different nuclei. We observed various morphotypes appearing in some of the evolved populations during the evolutionary experiment of the previous chapter and similar diversity of morphotypes among isolates from single patients. We speculated whether these morphotypes origin from the same hypha of a heterokaryon or from separately growing mycelia in the experimental populations and the patients. Heterokaryon compatibility testing suggests that all the morphotypes from a single patient and from a single experimental population are compatible, which indicates that heterokaryons are highly likely occurring in patients and in experimental populations, especially in patients with long- term of hyphal growth. Furthermore, we showed how the ratio of the different nuclei is flexible in the heterokaryon and can be used to adapt to the changing azole environments. These results highlight the importance of multiple interacting genotypes in *A. fumigatus*. The mixture of several genotypes in the hypha can play an essential role in the dynamic adaptation in both the patients and the field.

In **Chapter 6**, we investigated two compost–heaps, one of which contained azoles and one didn't. We showed that the azole-containing compost contains a larger genetic diversity of resistant *A. fumigatus* than the azole-free compost. This finding indicates that the azole-containing compost is a hot spot for the development and maintenance of azole resistant *A. fumigatus.* Importantly, we discovered a novel resistance mechanism with $TR_{46}{}^{3}$/Y121F/M172I/T289A/G448S that exerts pan- resistance to all medical triazoles. Infections with this novel mutant have been found in several patients in different hospitals in the Netherlands. The occurrence of $TR_{46}{}^{3}$/Y121F/M172I/T289A/G448S in strains of both mating types and the generation of $TR_{46}{}^{3}$/Y121F/M172I/T289A/G448S via sexual cross of two TR_{46} under laboratory condition suggest that the occurrence of sex in the compost is highly likely, however, more evidence is needed to confirm this. This finding underscores the fast development of resistant *A. fumigatus* strains and indicates a possible role of sexual reproduction in the development of azole resistance.

The thesis ends up with a general discussion **Chapter 7.** I discuss that the entire life cycle of *A. fumigatus* needs to be taken into account for understanding the emergence, maintenance and development of azole resistance in *A. fumigatus*. Asexual reproduction promotes the emergence of azole resistance via an increased mutation load; sexual and parasexual recombination shuffles the genes between genomes by generating genetic recombinants. Subsequently, the azoles act by creating various selection pressures on the genetically diverse genotypes. However, it is unknown whether sexual reproduction occurs in nature and whether that is indeed the driving factor for the development of TR_{34}/L98H and TR_{46}/Y121F/T289A mutants. In addition, the appearance of compensatory mutation and flexible heterokaryon are another two strategies facilitate

the persistence of azole resistance in *A. fumigatus*. I showed in this thesis that the role of all various aspects in the life cycles on the emergence of azole resistance is highly relevant and deserves further study. The ecology (such as compost hotspot) of *A. fumigatus* need be considered for in-depth research.

Nederlandse Samenvatting

Dit proefschrift toont hoe de levenscyclus van de schimmel *Aspergillus fumigatus* van belang is voor de ontwikkeling van azoolresistentie en de mogelijke evolutionaire routes die daartoe leiden. In dit werk wijs ik op het belang onze kennis van de schimmel biologie en evolutie voor het begrijpen van de ontwikkeling van azoolresistentie in schimmels. Ik concludeer dat azoolresistentie in *A. fumigatus* een gevolg is van selectiedruk door azool in de omgeving en de genetische variatie gegenereerd via verschillende aspecten in de *A. fumigatus* levenscyclus. Dit proefschrift introduceert ook een benadering met experimentele evolutie om de dynamiek en de mechanismen van de evolutie van de azoolresistentie te bestuderen. Daarnaast onderzoek ik welke omstandigheden in een omgeving kunnen leiden tot een mogelijke hotspot voor de ontwikkeling van resistentie. Tenslotte koppel ik hieraan de potentiele voorwaarden waaronder resistentie kan ontstaan en verspreiden in de longen van mensen en hoe dit afhangt van welk specifiek azool wordt gebruikt.

De introductie in **hoofdstuk 1** verstrekt gedetailleerde informatie over het probleem van resistentie ontwikkeling in *A. fumigatus* tegen azool antischimmel middelen. Azoolresistentie in *A. fumigatus* is uitgegroeid tot een wereldwijd probleem en belemmert de efficiëntie van klinische azool behandeling van aspergillose infecties in de longen (een ziekte die veel voor komt bij patienten met een slecht werkend immunsysteem). Algemeen wordt aangenomen dat er twee mogelijke routes zijn voor de ontwikkeling van azool-resistentie met een andere oorsprong van ontstaan: de klinische en de omgevings route. Dat betekent dat azoolresistentie kan ontstaan in patiënten behandeld met azool waardoor een selectie druk ontstaat, maar azoolresistentie kan ook al voorkomen in de omgeving waar *A. fumigatus* in contact komt met de patiënt. *A. fumigatus* komt veel voor in de natuur en leeft voornamelijk van dood plantenmateriaal. Hierdoor kunnen de verschillende azolen die worden gebruikt in de landbouw mogelijk leiden tot resistentie tegen medische azolen. Het belangrijkste resistentiemechanisme is aanpassing van het azool doelwitgen cyp51A, wat codeert voor een sleuteleiwit in de ergosterol productie van de cellen. De aanwezigheid van de zeer resistente mutantanten TR_{34}/L98H en TR_{46}/Y121F/T289A is toegenomen in de afgelopen jaren en wereldwijd verspreid. Er zijn diverse manieren van voortplanting bekend in *A. fumigatus*, echter is de complexe levenscyclus tot dusver verwaarloosd bij de studie naar de ontwikkeling van azoolresistentie. Verder is er weinig bekend over de ontwikkeling van azoolresistentie in milieu-isolaten op een evolutionaire tijdschaal. Tenslotte is er nog geen experimentele aanpak gebruikt om de verschillende agrarische azolen te testen op selectiedruk voor azoolresistentie.

Hoofdstuk 2 gaat over het huidige dilemma in het beheersen van *A. fumigatus* infecties bij patiënten: het stoppen van het gebruik van klinische azolen als resistentie ontstaat of de overgang naar nieuwe triazolen? Deze twee oplossingen zijn algemeen aanvaardbaar voor het tegengaan van de ontwikkeling van azool-resistentie in *A. fumigatus.* Door het

stoppen van het gebruik van klinische azolen, kunnen de resistente *A. fumigatus* stammen aanvankelijk lijden onder de kosten van resistentie die ze hebben in een azol vrije omgeving. Echter door de druk van deze kosten kunnen mutaties worden geselecteerd die de kosten van resistentie kunnen compenseren en dan leiden tot een resistent genotype dat geen extra kosten heeft in een azool vrije omgeving. We verwachten dus dat onderbrekingen in het gebruik van azolen niet zal werken tegen azool resistentie door deze compenserende mutaties. Ook overgaan op een nieuw azool variant zal de ontwikkeling van meer mutaties bevorderen en dit kan dan leiden tot overkoepelende resistentie tegen alle klinische azolen. In hoofdstuk 2, bespreken we ook een patiënt-dossier waar azool-behandeling heeft gefaald. Gedurende de azool behandeling, zijn culturen geisoleerd uit de patiënt met verschillende fenotypes (verschillende resistentieniveaus en morfotypen). De huidige twee behandel strategieën, kunnen door de compenserende mutaties of diversiteit van mutaties die ontstaan beiden mogelijk bijdragen aan het falen van de behandeling tegen *A. fumigatus* infectie.

De bevindingen van dit hoofdstuk verhogen onze zorgen over het beheersen van azoolresistentie in de klinische praktijk. Om dit probleem aan te pakken, is het belangrijk om te begrijpen door welk mechanisme resistentie ontstaat, hoe het kan verspreiden, en hoe resistente genotypes overleven.

In **hoofdstuk 3** hebben we ons gericht op de rol van de vegetatieve (ongeslachtelijke) voortplanting op de ontwikkeling van azool-resistentie. Eerder werd verondersteld dat vegetatieve sporulatie nodig is voor de expressie van azoolresistentie in *A. fumigatus* (Verweij et al. 2009), omdat de aseksuele sporulatie helpt de onstane resistente kernen te isoleren uit de meerkernige schimmel als één-kernige vegetatieve sporen, die kunnen uitgroeien tot een volledig resistente schimmel. We voerden een evolutie experiment met en zonder de rol van vegetatieve sporulatie. Vervolgens bleek dat de culturen met vegetatieve sporulatie een hoger resistentie niveau bereiken in vergelijking met het kweken zonder vegetatieve sporulatie. Wij concludeerden hieruit dat vegetatieve sporulatie een belangrijke rol speelt bij het ontstaan van azoolresistentie. Verder hebben we aangetoond dat azoolresistentie niet volledig dominant is in een heterogene meerkernige schimmel, dit sluit aan bij de hypothese dat vegetatieve sporulatie nodig is om te ontsnappen uit de meerkernige schimmel. Verder stellen we in een andere hypothese voor dat vegetatieve sporulatie de hoeveelheid beschikbare mutaties kan verhogen als gevolg van het grote aantal mitotische delingen tijdens vegetatieve sporulatie. De enorme hoeveelheden vegetatieve sporen geproduceerd verhogen het aantal aanwezige mutaties, en daarmee de kans op de aanwezigheid van sporen die azool resistent zijn. Deze ontdekking benadrukt het belang van dit specifieke deel van de levenscyclus dat leidt tot het ontstaan van azoolresistentie in *A. fumigatus*. Ook kan dit het vaker voorkomen van resistentie verhogen bij de behandeling van cavity aspergillose patiënten, waarbij vegetatieve sporulatie plaatsvindt verklaren. Bovendien bevordert vegetatieve sporulatie de verspreiding van *A. fumigatus*, wat de ruime verspreiding van de TR_{34}/L98H en TR_{46}/Y121F/T289A mutanten zou kunnen verklaren.

In **hoofdstuk 4**, bediscussiëren we de twee mogelijke routes waarop azool-resistentie kan ontstaan (klinische en omgevings-routes). Hiervoor deden we een evolutie experiment waarbij we een niet-resistente stam in aparte behandelingen blootgesteld hebben aan vijf verschillende landbouw- fungiciden, die in de afgelopen 30 jaar zijn gebruikt. We hebben getest of we de veelvoorkomende TR_{34}L98H resistentie mutant of andere zeer resistente isolaten op deze manier kunnen selecteren in het laboratorium. Hierna hebben we onderzocht of de geselecteerde resistente isolaten ook kruisresistentie hadden ontwikkeld tegen medische azolen. We toonden aan dat de dynamische trajecten van resistentie onwikkeling tegen de medische en agrarische azolen hoge gelijkenis vertonen, wat een sterke ondersteuning geeft aan de hypothese van een agrarische omgevings route naar resistentie. geevolueerde isolaten verschilden in resistentieniveau, groeisnelheid, kolonie structuur en sporulatie tijd. Er is echter geen TR_{34}/L98H mutant gevonden in dit evolutie experiment, maar we vonden een een mutatie G138S op cyp51A in één van de 36 lijnen. Onze resultaten leveren rechtstreeks bewijs voor een mogelijke omgevings route, in overeenstemming met eerdere studies, waarbij de hoog resistente TR_{34}L98H mutant kruisresistentie heeft tussen medische en landbouw azolen. Het is echter niet duidelijk of agrarische azool kan leiden tot de mutaties TR_{34}/L98H en / of TR_{46} /Y121F/T289A.

In **hoofdstuk 5** bestudeerden we de relevantie van de heterokaryotische toestand op de ontwikkeling van azoolresistentie in *A. fumigatus*. Een heterokaryon is een mycelium dat een mengsel van genetisch verschillende kernen bevat. De geevolueerde cultures van het evolutie experiment van het vorige hoofdstuk bevatten verschillende morfotypen. Een gelijksoortige diversiteit in morphotypen was ook gevonden bij verschillende isolaten uit één patiënt. We speculeren of deze morfotypen hun oorsprong hebben in één individueel heterokaryotisch mycelium of in afzonderlijk groeiende mycelia voor de experimentele cultures en de patiënten. Heterokaryon compatibiliteit tests tonen dat alle morfotypen van één enkele patiënt en ook die uit één onderzoekspopulatie compatibel zijn, wat aangeeft dat heterokaryons zeer waarschijnlijk voorkomen bij patiënten en in de experimentele populaties, vooral bij patiënten met een langdurige groei zonder sporulatie. Bovendien toonden we aan hoe de verhouding van de verschillende kernen variabel is in het heterokaryon en kan worden gebruikt door *A. fumigatus* om zich aan te passen aan de aan of afwezigheid van azool in het groeimedium. Deze resultaten benadrukken het belang van de interactie tussen genotypen in *A. fumigatus*. Het mengsel van verschillende genotypen in een mycelium kan wellicht een essentiële rol spelen bij de dynamische aanpassing zowel in de patiënten als in het veld.

In **hoofdstuk 6** onderzochten we twee compost-hopen, waarvan één azolen bevatte en één niet. We toonden aan dat de compost met azool een grotere genetische diversiteit van resistente *A. fumigatus* huisvest dan compost zonder azool. Deze bevinding geeft aan dat het azool bevattende compost een hotspot is voor de ontwikkeling en instandhouding van azool resistente *A. fumigatus*. Verder is een belangrijke ontdekking in de compost dat er een stam met een nieuw resistentiemechanisme (TR_{46}^{3}/Y121F/ M172I/T289A/G448S) dat resistent is tegen alle beschikbare medische azolen. Infecties

met deze nieuwe mutant zijn gevonden in verschillende patiënten in verschillende ziekenhuizen in Nederland. Het optreden van TR_{46}[3]/Y121F/ M172I/T289A/ G448S in stammen van beide mating types (parings typen) en het genereren van TR_{46} [3]/Y121F/ M172I/T289A/G448S via seksuele kruising van twee TR_{46} onder laboratoriumomstandigheden suggereren dat het voorkomen van de seksuele cyclus van *A. fumigatus* in compost zeer waarschijnlijk is. Echter, meer bewijs is nodig om dit te bevestigen. Deze bevindingen onderstrepen dat de snelle vorming van resistente *A. fumigatus* stammen en wijzen op een mogelijke rol van de seksuele voortplanting hierbij.

Het proefschrift eindigt met een algemene discussie **Hoofdstuk 7**. Ik bediscussieer dat de gehele levenscyclus van *A. fumigatus* nodig is voor het begrijpen van het ontstaan, het behoud en de ontwikkeling van azoolresistentie in *A. fumigatus*. Vegetatieve voortplanting bevordert het ontstaan van azoolresistentie via een verhoogde hoeveelheid beschikbare mutaties; seksuele en parasexuele recombinatie wisselt de genen uit tussen genomen door het genereren van genetische recombinanten. Vervolgens zorgt de aanwezigheid van azolen voor verschillende selectie druk op de genetisch diverse genotypes. Het is echter niet bekend of seksuele voortplanting wel in de natuur voorkomt en of dat inderdaad de drijvende factor voor het ontstaan van de zeer resistente TR_{34}/L98H en TR_{46}/Y121F/T289A mutanten is. Bovendien, zijn het optreden van compenserende mutaties en de flexibiliteit van een heterokaryon nog twee strategieën die kunnen bijdragen aan de aanhoudende azoolresistentie in *A. fumigatus*. Ik heb in dit proefschrift laten zien dat de rol van alle verschillende stadia van de levenscyclus zeer relevant zijn voor het ontstaan van azoolresistentie en dat er meer onderzoek nodig is om precies te begrijpen hoe de hele levenscyclus het ontstaan van resistentie beinvloed. De ecologie (zoals in een compost hotspot) van *A. fumigatus* zal ook meer diepgaand onderzoek nodig hebben om een oplossing te vinden voor de groeiende azool resistentie.

摘要

本论文研究了烟曲霉生活史与耐药产生的相关性以及可能的耐药进化途径。该论文突出了真菌生活史以及进化在理解唑类耐药的发展中的重要性。唑类耐药是基于生活史产生的遗传多样性的基础上唑类药物高效选择的结果。本文采用了遗传进化试验方法研究唑类耐药演化的动力和机制。结合跨学科（遗传、进化和真菌学）知识探索耐药产生的原因和传播条件，我们并比较了临床唑类施用的程序以及宿主感染环境对烟曲霉耐药发展的影响。

第一章介绍了关于烟曲霉唑类耐药的详细信息。烟曲霉唑类耐药严重阻碍了临床曲菌感染治疗的效率，并已经成为一个全球性问题。人们普遍认为有两种可能的唑类耐药发展途径：临床和环境的途径。主要的耐药机制是靶基因-编码麦角甾醇途径中的关键酶 cyp51A 的突变。感染高抗性菌株 TR_{34}/L98H 和 TR_{46}/Y121F/T289A 的病例逐年增加，进而在全球蔓延。烟曲霉拥有多样化的生活史（有性、无性和拟性生活史），但是，人们忽略了这种复杂的生活史对于理解唑类耐药发展的重要性。此外，环境途径的耐药性的发展途径假说也缺乏直接的实验证据，农业杀菌剂在耐药发展的差异也有待于进一步的研究。

第二章讨论了目前临床上烟曲霉耐药性管理上的窘境：停止使用唑类或更换新的三唑类？在临床上，这两种解决方案通常被采用用于控制病情恶化。然而，停止使用临床唑类， 耐药的烟曲霉将会承受耐药损失，进而产生补偿性突变。然而， 更换新的药物，将会导致更多的突变，最终多耐药性菌株的产生。在本章中，我们提供了一治疗失败的病例，由于在治疗过程中采用两措施，补偿性突变和多耐药性突变可能恶化了烟曲霉感染的治疗。本章节唤起对临床烟曲霉耐药性管理的担忧，寻求有效管理烟曲霉耐药性的解决措施破在眉睫。因此， 探索耐药性发生、传播、持久机制是很重要的措施之一。

在**第三章**中，我们探索了无性繁殖对唑类耐药性发展的影响。基于无性繁殖产孢子过程利于耐药性的表达假说，（VERWEIJ 等，2009），即无性繁殖产孢子过程利于耐药型性菌核从众多的敏感性菌核中释放成独立的耐药性菌株，我们建立并比较了有无产孢过程的进化试验。试验结果证明，与无产孢子过程相比，产孢子过程产生了更高耐药性的菌株。我们证明了无性繁殖产孢子过程促进了耐药性的出现。不完全显性的抗性也支持了产孢子过程助于耐药性菌核逃离敏感菌核菌丝的假说。此外，我们还提出了另一种假说，即无性繁殖产孢子过程可以增加突变量，由于无性繁殖产孢子过程经历大量的有丝分裂。这一发现强调了烟曲霉无性生活史对耐药性发展的重要性，同时这一发现有助于解释有产孢子过程参与的腔曲菌病病人，在治疗过程中耐药性不断增加的现象。最后，产孢子过程也利于烟曲霉的传播，导致高耐药性菌株 TR_{34}/L98H 和 TR_{46}/Y121F/T289A 的全球性传播。

第四章中，针对有争议的烟曲霉耐药性的发展（临床和环境的途径），我们设计了遗传进化试验，暴露敏感的烟曲霉于 5 种农用杀菌剂下，我们欲检测环境耐药性的途径的可能性，暴露于农用杀菌剂是否导致对临床药物的抗性。在遗传进化过程中， 我们发现，敏感菌株进化获得不同的抗性， 菌丝生长速度，菌落结构以及不同产孢时间。虽然没有发现广泛流行的高耐药性 TR_{34}/L98H 突变体，但是我们 36 个进化组里发现了一个 cyp51A-G138S 突变体。对临床药物和农业杀菌剂唑类的动态进化轨迹表现出很高的相似性，这一发现极大支持了环境耐药性进化假说。我们的研究结果提供可能的环境耐药性途径的直接证据，与以往的研究，高抗 TR_{34}/L98H 交叉抗性于临床药物和环境农业杀菌剂唑类是一致的。但是，目前尚不清楚环境杀菌剂唑类是否能够诱导产生 TR_{34}/L98H 或 TR_{46}/Y121F/T289A。

第五章中，结合第四章的进化实验中的多形态烟曲霉，并从临床患者多株烟曲霉的观察中，我们欲检测是否菌丝异合体参与烟曲霉唑类抗性的发展中。异核体菌丝伴随参与真菌生活史的各个阶段。在本章中,我们研究了异核体与烟曲霉唑类耐药性的相关性。异核体相容性试验表明，所有这些多形态菌落是可兼容性的，这表明异核体很有可能发生在患者和实验室的长期菌丝生长阶段。此外，试验显示异核体是如何灵活地适应不断变化的唑类环境。此研究有助于我们理解异核体在野外以及病人体内动态的抗性适应性。相关的异核体进一步形成二倍体以及单倍体化这些潜在的对抗性发展的研究有待于更进一步的探索。

在**第六章**中，我们调查了唑类污染和有机两种堆肥中烟曲霉的种群情况，结果表明，唑类污染的堆肥中包含三种烟曲霉基因型：野生型(4%)，抗性 TR_{46}/Y121F/T289A (92%)和新突变体 TR_{46}^{3}/Y121F/M172I/T289A/G448S(4%)，远远大于绝大多数敏感性为主的有机堆肥(98%)野生型,2% TR_{34}/L98H 。启动字进一步延长的新突变体 TR_{46}^{3}/Y121F/M172I/T289A/G448S 对所有的临床药物均具有抗性.同时,此突变体已经感染荷兰各地医院的数位病人,引起严重性感染,甚至死亡。新型突变体 TR_{46}^{3}/Y121F/M172I/T289A/G448S 存在两种交配型，并且在实验室条件下两个 TR_{46} 的有性杂交产生 TR_{46} ^{3}/Y121F/T289A 的试验表明有性生殖可能对产生新型的更抗性的烟曲霉起着巨大的作用。然而是否有性生殖可以在野外发生需要更多的证据.但是新型突变体的发现引起我们对抗性快速发展和临床治疗的担忧。

第七章以对整个论文的讨论结束本论文。人类机遇性病原菌抗性研究是一个复杂的问题,因为烟曲霉多样性的生态位,以及多样化的生殖方式提供了烟曲霉面对唑类压力的具备条件.烟曲霉多样化的生活史,生态热点位以及唑类的抗性筛选等因素需要考虑到理解烟曲霉唑类抗性的发生,发展以及持续。这样方可制定有效的,具有针对性的预防烟曲霉唑类抗性发生,传播以及感染度的措施。

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At this moment, firstly, I want to say to myself, “congratulations, Jianhua, you made it, you are a very brave girl, you should be very proud of yourself.”

Indeed, from a little Chinese girl, travelling far away from eastern China, now you became into an independent PhD girl. This is a very big step for you, and it was not that easy at all.

At the age of 26, you decided to turn your life upside down and inside out. With your poor English, you managed to arrive to Wageningen safe and sound. You were not afraid at all, even though you never went abroad ever before. Even at the moment the bus driver dropped you off somewhere not matching with the stop you wanted. A little Chinese girl pulled a huge suitcase along the wild fields and tried to ask directions all the way and finally arrived at the Bornsesteeg. But Wageningen left a very good first impression on you at that first arrival. Fresh air, sometimes mixed with horse poop and green grassland with ponies. You started to enjoy these surroundings and breathed the fresh air along the way home (indeed it is much cleaner than Beijing’s air).

The second day after you arrived, you started your project. You started a new life in a completely unfamiliar environment. You even don’t know who are your colleagues, so you accidentally joined the EPS PhD symposium in Utrecht. But you grew very fast, you made new friends and got into your project more and more after half a year. Yes, after four years you are not the little struggling girl any more, you are a very brave and independent girl. You have achieved and succeeded using the “weird Chinese logical way of thinking” --- not all Chinese logical thinking fits here, e.g. China is big, The Netherlands is small, everything is very different except that the sun rises from east and sets from west, but still with 6/7 hours delay..... :). But it is not that bad to think about the Dutch problems with the Chinese way of thinking. :) :)

Bas, Sijmen, Fons, I appreciate all help from you guys very much. You are the witness of my changes over these four years! Even though so far I am not sure who is my major supervisor, I am so happy that each meeting could stimulate you guys to lots of brainstorming. You are the best supervisor group ever. Sijmen acts like my big brother, especially at the beginning you gave me a lot of support. You are so patient to guide me and show me around the lab the first time.

Fons, I would like to name you as my older friend. A magic and creative person, full with lots of ideas, but I could not always understand what you are talking about due to my poor knowledge at the beginning. I am very happy that I could communicate with you about everything so freely over four years of study. :) :) What a success! Every time, I come out from your office, sky became much bluer even when it is a raining day! Fantastic! Thanks for lots of leisure talk, and a “romantic walk” (you drived the car and I cycled). When you tell me your phone is blocked, I could not stop laughing, sorry for that. I have never done this ever before, “laughing at” the supervisor in front of the supervisor, that is so amazing!!!. Discussing with supervisor when I eat apples, so relaxed discussion, I never did that before.

Bas, you are the first person I contacted when I applied for this subject. Thanks you for initiating this very interesting project. I am super happy with the subject so far and with it growing so fast. There are more and more collaborations and new projects coming in. I believe our lab will be the aspergillus center one day! :) :) I was so surprised with your height at my first sight, it means I have to lengthen my neck and tilt my head to talk to you every time, and it is painful :) :) :).

Paul, thanks very much for your expertise. You are my clinical dictionary. It was so amazing that we could talk via email with around 10 emails per day back and forth. Also thank you for all clinical materials that you provided me. Willem, a humorous guy, I like your attitude, and love to make jokes with you. First time we met, I said my English was not good, you said that your Chinese was not good either. Thanks for your kind encouragement. Another thing, I want to ask you, at our first meeting you told a story about a guy who works with azole resistance but could not pronounce azole well, I wonder what is your feeling when you hear my way of pronouncing azole :) :) :) :) . Must be fun, right?

Thanks so much for this amazing supervisors team group, it is because of you guys, I fell deeply in love with my subject, and it is so much fun in science. I love it! Fantastic idea! Ingenious design! Super funny fungi! I enjoyed so much to work with you guys!

At the same time, we also buildup huge network and collaborate with CLM (Duurzame landbouw -Gezond voedsel - Vitaal platteland), National Institute for Public Health and Environment (RIVM) and Praktijkonderzoek Plant & Omgeving Bloembollen, Boomkwekerij & Fruit. Thanks Anton Rietveld (RIVM), Jeanne van Beek & Peter Leendertse (CLM, experienced advisor sustainable cultivation), and Henk Gude (PPO) for all your expertise. Every time we have a great discussion and new inspiration. Thanks Peter Leendertse for analyzing the azole concentration in the compost. Thanks Henk Gude and André Conijn for showing us the tulip field and helping us collecting all samples. Also thanks Jacques Meis and Ferry Hagen from the Department of Medical Microbiology and Infectious Diseases, CWZ Hospital for contribution of discovery of new resistance mutation. Lastly, also thanks many clinical advisors from Dutch national surveillance programme for screening the new mutations. Lastly, thanks Prof Wei Liu from the Peking University First Hospital Research Centre, and Prof Li Han from Institute for Disease Control& Prevention, AMMS of China for supporting us to collaborate with China.

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followed your step and start my project. Thanks for your support and lots of discussion. From now on, we can discuss more and I believe we will be a great Aspergillus girl team.

Bertha, and Marijke, our great technicians, without you, our lab could not run smoothly. I learned a lot from you two. Also thanks for showing your grandbabies to me. I am so happy to talk with you. Thank you Bertha for giving me lots of comforts when I am disappointed. I still remember that big hug from you after my crying. Gabriella, thank you so much for your great company. I like chatting with you. You always encourage me when I feel sad. You are a great listener and care people's feeling. Thanks for sharing your life story with me. Thanks for your great ladybug, I do take care of them very good.

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Claudio, my communism friend, we started more or less at the same time. We went to conferences and travelled together. We had lots of great times together. One day I was so surprised I have more pictures with you than with Eric :) I like your smiling face on every picture. I enjoyed chatting with you and thanks for teaching me how to play poker (that magic heavy box with colored chips), and also the sequence game, which I feel I am kind of good at.

Lidia, it is so nice to have you as a friend, I love to have girl talk with you and Vivian. We all are so excited to talk, it puts smiles on our faces. Thanks for your showing me the belly-dance. It was such great fun to shake my hip continuously, even the day after my neck felt painful..... strange :). I was so enjoyed by your belly dance, amazing...., let us dance next time again! :) :)

Alex, my tablemate, roommate, colleague, friend..... it is so difficult to give a conclusion about our relationship. We "fight" quite some times, but all ends in a good way. It is quite amazing we did not become enemy even at the situation that we have to meet 16 hours per day (more or less). But on the other way around, we become closer and closer. I was touched by your behaviour when you helped me complain to Duur just because I was upset. Hey, buddy, you do care about my feelings :) :) :) . Thanks Alex, I am very happy especially after you become postdoc, you try to make different food every day and let me try as well. I quite enjoyed all food, tasty soup (I guess I am addicted to it now) and deserts. And funny thing, I taught you 谢谢, you replied 谢谢谢。Speechless :):) . By the way, I want to watch the stars with you one day..... you promised to me!! Last thing, thanks for your beautiful drawing on my balloons, I love them.

Anna, thanks so much for introducing Russian culture to me, tasty beef soup, cutest Matrioska (my favourite gift, small rat stay inside of cats, isn't that amazing?)

Vivian, my best Greek friend, even though I only know one Greek. :) Thanks for your company and sharing all happiness and sadness with me. We had a lot of fun at the bar, at my home playing Games, eating Chinese food and Greek food. I was so touched that you came from Utrecht and cooked big cheese cake and bean soup for us which was enough to eat for a few days, amazing cook. :) :)

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Guiling, do you still keep the tiger and donkey from Winnie the Pooh? I still remember how crazy we were when we saw lot of cute dolls in the market. Shopping girls nonstop shopping :) :) . By the way, thanks for your very useful suggestion on designing the vase and autumn leaves in my room. I like your room. :) If one day you open a designing company, please do not forget to employ me :) :) Come on, Guiling!!!

Tao, are you still thinking to form a music band? :) (Guiling, Yanli, me, we are so happy to sing to music that you played on the guitar). We felt 10 years younger when we sang these university songs. One day, when you want to form a band, please don't forget to ask us. :) :)

Yanli, thanks for bringing mahjong all the way from China, can you imagine how wonderful it is to play mahjong outside of China. Full of laughers at my home, Junwei was busy with counting the dots on the mahjong whole night, Defeng cheated to win..... hope you guys enjoyed a lot with mahjong. Let us play again.

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great piano music. Thanks for your simultaneous Translation of Dutch talk for me. So sweet!

Alex, Claudio, Valeria (sorry so far I could not pronounce your name 100% correctly), Vivian, Cris, Florien, Tina, Lennart, Kritty, Margo, Anneloes, Kim, Pingping, Justin and Joost. All my tablemates, thanks for your company, snack sharing, Chinese dinner night, movie night, poker night, Picnic on the river bank, cycle in the forest, mahjong night, Guito night, Greek night, steps party, beer night. I had so much fun to stay with you guys. Oh, Valeria, you could watch star at your place, it must be amazing, right..... like "little prince"....:) :) Cris, a very good runner, thanks for sharing your marathon stories with me, funny snack and running golden medal! Ramon, I like your banana tree nearby you, wish it will fruit one day. Thanks for your provide all nutrient for my plants, even it did not bloom at all after our lots of efforts. Lennart, enjoying reading a paper stained with coffee, which could keep you awake. Kritty, thanks for experiencing a typhoon with me in the campus, we survived. I am still imaging how you try to escape the strong wind, have to stop by a pole in the middle of nowhere (sound like a Pole-dancing girl in the strong typhoon of the Netherland). Is that cool? Margo or mango? Both are tasty, thanks for inviting us for concert, we enjoyed a lot, fantastic group. Thanks for Tania and Andre for the tasty Portugal tea and amazing Bacalhau.

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Andy, my new tablemate for a whole, I feel a little close to you maybe because we are both from far away, :) :) :) like a warm lunch. :) :), and New Zeeland can grow the Chinese kiwi in the best way. Marjon, awesome princess; Diego, an experienced dancer. You are an amazing musician team, so lovely group!

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Bareld, my garden neighbour, thanks for your company as well. I enjoyed all leisure talks with you! Thanks for your tasty berry and taking care of my garden when I am away!

Thanks for sharing your rich life experience with me! By the way, how your cats are doing? I was surprised you have never spent longer holidays because of your cat. So I hope you can have a longer holiday one day!

I appreciate the understanding and support from my family in China very much. I am so sorry I could not attend lots of important celebrations. Thanks to my dear sister Jianhong (建红) and brother- in law Yuanyuan（元元） for taking care of our parents when I am away. Thanks to my nephews Dacong(大聪), Ercong（二聪）, Jingming（婧茗）for bringing lots of fun for my life. Thanks to my dear brother Xue (雪)and sister- in law Dandan (丹丹) for encouraging me when I confront the difficulties abroad. Thanks to my dear friends 二牛，莎莎, 俊刚 for your great support! I wish all of you have a great happy life ahead.

I want to thank all Eric's family: Carla, mama Tineke, papa Lauke, Marcel, Lauke, Anneke, Lukas, Peter, Marissa, Kasper, and Karlijn. Thanks for all of you accepting me and welcoming me as a family member. I always do feel family love from you guys, we went camping together, de tour of France, football match, purple flower park, boating, fireplace night, sinterklaas and car racing.....I think I never can forget these amazing moments ever.

Eric, what do you want hear from me? :) :) probably too much to write down, let us talk in bed :):). However, I will give you a short summary: thank you for bringing endless happiness to me. I feel endless love from you. My cook, my lover, my driver, my boy!

Curriculum Vitae

Jianhua Zhang was accidently born on 25th of Jan 1985 in Jiaocheng, Shanxi, China. She finished her high school education in 2004 in Jiaocheng Secondary High School. The same year, she started her 4 years of Bachelor education majored biology in the Taiyuan Normal University, there she was trained to be a good Biology teacher, and she fell in love with biology since then. Therefore, after Bachelor graduation in 2008, she decided to leave the home town and broaden her view. She began her Master education in one of the hottest cities in China:Wuhan, Central China Normal University, where she worked in the Hubei Key Laboratory of Genetic Regulation and Integrative Biology & Key Laboratory of Pesticide and Chemical Biology. She studied the fungicide resistance of post-harvest pathogen *Penicillium digitatum, italicum & Ustilago maydis* and developed novel and effective antifungals under the supervision of Professor Deli Liu. Since then she realized she was able to do laboratory work and publish the work as well, although she could graduate as a Biology teacher as well. However, she decided not to become a Biology teacher like other classmates did after attaining the Master degree. This time, she made a huge decision in her life; she made a decision to go abroad to get a PhD degree. On last day of March in 2012, she flew to the Netherlands and started her PhD life. During these four years, she tried to figure out the importance of *Aspergillus fumigatus* life cycles in the emergence of azole resistance and the azole resistance evolution from a unique perspective of genetics, evolution and fungal biology. She linked and managed her five supervisors that are promoters Prof. Dr B.J. Zwaan & Prof Dr. Paul E. Verweij, co-promoters Dr.ir. A. J. M. Debets & Dr S. Schoustra; and external supervisor Willem J. G. Melchers. In this thesis, her adventures during that PhD are presented.

1. Paul E. Verweij[*, 1], **Jianhua Zhang**[*, 2], Alfons J.M. Debets[2], Jacques F. Meis[1, 4], Frank L. van de Veerdonk[3], Sijmen E. Schoustra[2], Bas J. Zwaan[§, 2], and Willem J.G. Melchers[§, 1] In- host adaptation and acquired resistance in *Aspergillus fumigatus*: on the horns of a dilemma. ***Lancet Infectious Diseases*** accepted (2016).
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4. **Jianhua Zhang**, Li Zhao, Jie Zhang, Rui Han, Shuxiang Li, Yongze Yuan, Jian Wan, Wenjing Xiao, Deli Liu.Optimised expression and spectral analysis of the target enzyme CYP51 from *Penicillium digitatum* with possible new DMI fungicides. ***PEST MANGMENT SCI*** (2010) 66: 1344–1350.
5. Rui Han, **Jianhua Zhang**, Shuxiang Li, Shufen Cao, Hui Geng, Yongze Yuan, Wenjing Xiao, Shenghua Liu, Deli Liu. Homology Modeling and Screening of New14α-Demethylase Inhibitor (DMI) Fungicides Based on Optimized Expression of CYP51 from *Ustilago maydis* in *Escherichia coli*. ***J. Agric. Food Chem.*** (2010), 58, 12810-12816.
6. Xueting Yang, Yongze Yuan, Junzhong Yang, **Jianhua Zhang**, Shangying Xu, Kehan Xu, Lixia Zeng, Deli Liu. Over-Expression and Purification of Recombinant Methyl Parathion Degrading Enzyme by Lactose Induction. (2010) (*EI& EPPH*).

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)

- Failure of posaconazole therapy in a renal transplant patient with invasive Aspergillosis due to *Aspergillus fumigatus* with attenuated susceptibility to posaconazole (2011)

Writing of project proposal (4.5 ECTS)

- Understanding azole resistance in *Aspergillus fumigatus*: condition-dependent mutations for resistance and compensatory evolution

Post-graduate courses (3.6 ECTS)

- Introduction to R for statistical analysis; PE&RC (2013)
- Evolutionary biology workshop; Urbana (2014)

Laboratory training and working visits (0.3 ECTS)

How to work with *Aspergillus fumigatus* in the lab safely and how to prevent *Aspergillus fumigatus* contamination; Medical Microbiology, Radboud University Medical Centre, Nijmegen (2012)

Deficiency, refresh, brush-up courses (1.5 ECTS)

- Basic statistics; PE&RC (2013)

Competence strengthening / skills courses (4.5 ECTS)

- PhD Competence assessment; WGS (2012)
- Techniques for writing and presenting a scientific paper; WGS (2013)
- Improving writing; Wageningen into languages (2013)
- Scientific writing; Wageningen into languages (2014)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.8 ECTS)

- PE&RC Weekend (2012)
- PE&RC Day (2012, 2014, 2015)

Discussion groups / local seminars / other scientific meetings (9 ECTS)

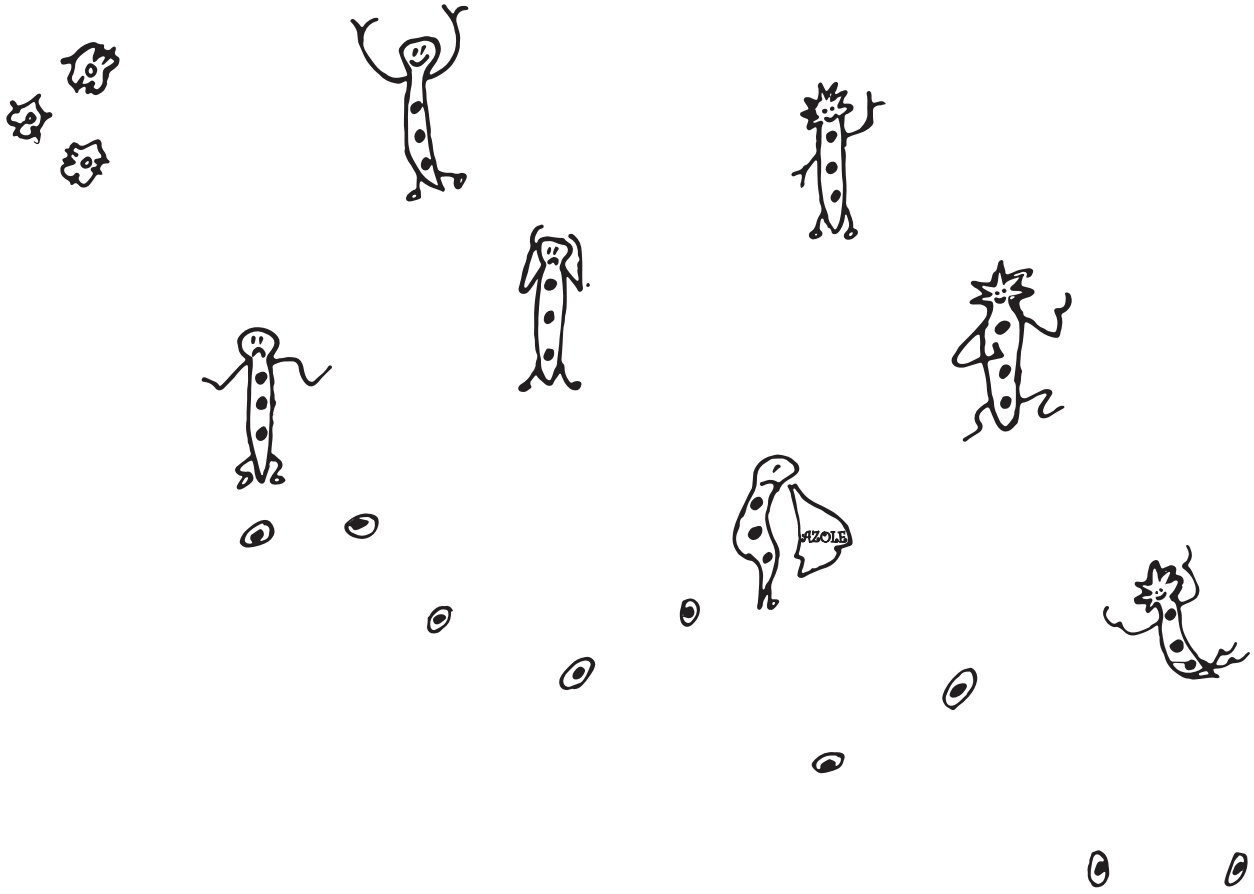
- Experimental evolution discussion group (2012-2015)
- The annual meeting of the section mycology of royal (KNVM, CBS) (2012-2015)
- WEES Seminars (2012-2016)
- Netherlands Annual Ecology Meeting (2013)
- Evolution in the laboratory symposium (2013)
- Banana day (2014)
- World without pesticides (2015)

International symposia, workshops and conferences (14.9 ECTS)

- Fungal genetics conference (2015)
- ESEB Congress (2013)
- European meeting of PhD students in evolutionary biology (2014)
- Aspergillus satellite meeting (2015)
- Resistance and persistence in *Aspergillus fumigatus*; KNAW (2015)
- 13^{e} European conference on fungal genetics (2016)
- Aspergillus satellite meeting of ECFG (2016)

Lecturing / supervision of practicals / tutorials (14.4 ECTS)

- Molecular and evolutionary ecology (2014, 2015)
- Genetics analysis tools and concepts (2015)



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